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REFERENCES: 1. Welling, A.; Watkins, W.W., and Raines, S. L.: J. Urol. *77*:773, 1957. 2. Thompson, I. A., and Amar, A. D.: J. Urol. *82*:387, 1959. 3. Lippman, R. W.; Wrobel, C. J.; Rees, R., and Hoyt, R.: J. Urol. *80*:77, 1958.

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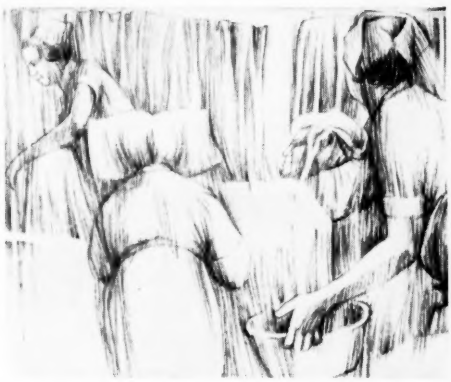

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John R. Park

FOREWORD

This volume is a Festschrift presented to John Rodman Paul by his students and colleagues throughout the world, as an expression of affection and esteem. It marks his retirement on 30 June 1961, as Professor of Preventive Medicine at Yale. Happily this retirement does not mean withdrawal from the scene of action, for on 1 July 1961, he became director of the World Health Organization Serum Reference Bank, located in the Department of Epidemiology and Public Health at Yale. His new laboratory is one of three recently established centers (the other two being in Johannesburg and Prague) for the development of serologic epidemiology on an international scale. Thus he is continuing a career at Yale which began some 33 years ago, when he came to New Haven at the invitation of Dr. Francis G. Blake, then chairman of the Department of Medicine. He was first appointed assistant and later associate professor of Medicine. In 1940, the Section of Preventive Medicine was created within the Department of Medicine, with Dr. Paul as professor and chairman.

John Paul's contributions to medicine cover a wide range, represented to some degree by the titles of the papers in the present volume. Although the subjects to which he has devoted himself have been diverse, there has been a unifying theme in his approach to them. This arises from a philosophical turn of mind which very early caused him to view the problems of disease in the long perspective. From the beginning of his career he held the conviction that "clinical medicine means more than the practice of the technics of diagnosis and therapeutics, for, although it deals primarily with the care of the sick individual, it is also concerned with *the whole subject of disease in living people*".[1] In 1938[2] he proposed the term *clinical epidemiology* — "for a new science, — concerned with circumstances, whether they are 'functional' or 'organic', under which human disease is prone to develop. It is a science concerned with the ecology of human disease. But it is more than that, for any science worthy to be qualified by the name Clinical, should involve some attempt at the interpretation of the circumstances with which it deals. It must face the quetsion 'why', as well as 'how'."

This approach, as he has so often stressed, is equally applicable to infectious and non-infectious diseases; its usefulness "lies not only in the discovery of new intrinsic or extrinsic factors, which may be found either indoors or outdoors, but in the discovery of new concepts. This includes the

[1] Paul, J. R.: Preventive Medicine at the Yale University School of Medicine. Yale J. Biol. Med., 1940, *13*, 253-258.
[2] Paul, J. R.: (President's Address) Clinical epidemiology. J. Clin. Invest., 1938, *17*, (No. 5) 539-541.

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concept of certain new etiologic forces which lie back of those which were once thought to be basic such as, for instance, the factors which lie back of the pneumococcus as a cause of pneumonia. Of late years conservative opinion does not allow anything to be really considered as 'etiology' unless we can succeed in getting it into a test tube, unless we can precipitate it, —unless we can crystallize it as it were. This is due, of course, to our current methodology which has, perhaps, become more of a religion than most of us realize. I think it may have led to a slightly narrow interpretation of clinical investigation on our part, for clinical investigation certainly should be given the opportunity to spread itself up into philosophy, if it will, as well as down into the basic sciences."

In the twenty-three years since these words were written, progress has been made; with considerable struggle, epidemiology is coming into its own as a basic and a clinical science, and its potentialities, forseen so clearly by Dr. Paul, are gradually being realized. During these years, he has been in the thick of the battle as an investigator and teacher, organizing and carrying out both frontal and flank attacks on various epidemiological problems at home and abroad. A year and a half ago, he had occasion to dictate an informal history of research in the Section of Preventive Medicine, with some notes on earlier work carried out while he was assistant and associate professor of Medicine. The transcription of this dictation he has never seen. Nevertheless, we are taking the liberty of presenting it here, without his knowledge (and with only minor editing) as a personal record of the pleasures of scientific exploration and discovery in the mid-20th century by a physician, a clinical epidemiologist, a naturalist, and a philosopher,—viz. J.R.P.

DOROTHY M. HORSTMANN
(*Guest Editor*).

FROM THE NOTEBOOK OF JOHN RODMAN PAUL

"At an earlier date, some four or five months ago, I dictated an outline of the activities of the Department of Medicine during the 1920's and '30's with emphasis upon the work of Blake and Trask on scarlet fever, their recognition of the scarlatinal toxin, and the use of antisera in the therapy of the disease. This was at a time when scarlet fever was a much more serious disease than it is at present. The report also emphasized Dr. Blake's work on pneumococcal pneumonia, his discovery and classification of pneumococcus variance, and later a great deal of work on the therapy of pneumonia, both by pneumothorax and later by chemotherapy and antibiotics.

From the work on scarlet fever, a disease of streptococcal origin, a number of lines of research developed. One of them included the studies on the epidemiology of rheumatic fever, which began before the role of the hemolytic streptococcus was appreciated. These studies utilized the family approach. On the basis of the way rheumatic fever spread through families, it was concluded that respiratory infection of some kind precipitated the acute attack of the disease. Rheumatic fever studies covered a period of at least eight years. During this time many of the environmental and social aspects were explored through intimate, long term studies on all members of 122 rheumatic families, and suitable control families. Some sixteen years later in 1947 many of the families were re-studied by Dr. Freida Gray and Dr. Robert Quinn.

DISCOVERY OF THE HETEROPHILE ANTIBODY TEST FOR THE DIAGNOSIS OF INFECTIOUS MONONUCLEOSIS

The events which led to the discovery of this diagnostic test may be of some interest. In 1929, Davidson of Chicago published a paper indicating that agglutinins to sheep cells were present in cases of serum sickness and he elaborated on the fact that this was an example of the Forssman antigen which had been demonstrated in 1911. It had been found that cells obtained from the organs of many animals when injected into an appropriate animal, such as a rabbit, were capable of giving rise not only to specific antibodies but also to nonspecific antibodies and these were demonstrable in varying degree in the form of hemolysins and agglutinins for sheep cells. Serum sickness was a very common disease in the New Haven Hospital during the period between 1928 and 1930, the widespread use of serum for cases of scarlet fever and for pneumonia providing plenty of clinical material. Davidson's results were quickly confirmed at the Yale Laboratory. At the same time, a study on rheumatic fever was going on, and the question arose as to whether the similarity of symptoms between rheumatic fever and serum sickness might not make it worthwhile to look for this heterophile antibody in cases of rheumatic fever. Such a search was started but we were unable to demonstrate a consistent or appreciable increase in the heterophile antibody content of the serum of patients suffering from rheumatic fever.

In the course of this study however, a number of controls from individuals suffering from serum sickness and a variety of other clinical conditions were assembled. Quite by accident it was discovered that antibodies capable of agglutinating sheep cells were present in the specimen of serum of a patient ill with infectious mononucleosis. The titer of antibody in this

serum was much higher than had been described in serum disease or in any other clinical condition which we had studied. The man who was collecting the specimens of serum from the "control" patients was Dr. W. W. Bunnell, now a well-known practitioner of medicine in Farmington, Connecticut, and the patient on whom this discovery was made was a medical student, Mr. Franklin Foote, now a physician, and Commissioner of Health of the State of Connecticut. The finding seemed to be unusual and something of a fluke. It was not until a good seven or eight months later that another case of infectious mononucleosis came to our attention; this time also it was a medical student with infectious mononucleosis and jaundice and here again a high heterophile antibody titer was demonstrable. Gradually a series of infectious mononucleosis cases was assembled and it seemed that this was a fairly characteristic finding. The first paper, "The Presence of Heterophile Antibodies in Infectious Mononucleosis," was published in 1932, and covered a series of some 275 general hospital patients in which the test had been carried out, mostly with negative results, with the striking exception of the positive findings in the cases of infectious mononucleosis. That the test would be useful in diagnosis was established essentially by Dr. Bunnell, who in the subsequent year gathered a much larger series of cases. In view of the simplicity of the test it was not long before reports began to appear from many laboratories and almost immediately it was pointed out that the type of antibodies present in infectious mononucleosis differed from those present in serum disease, and the two could be differentiated by absorbing the serum with ox cells or guinea pig kidney. Some of these papers criticized the Yale workers for calling this a heterophile antibody, and a lively discussion appeared in the medical literature. One of the valuable letters in the archives of this study was that received from Nobel Laureate Carl Landsteiner, who wrote that he was in real sympathy with our calling this a heterophile antibody regardless of the critics. As a postscript to the finding it may be said that although the test is widely used today, the etiology of this disease remains an enigma.

THE YALE POLIOMYELITIS STUDY UNIT

The story of the work on poliomyelitis begins in 1931. This was undertaken jointly by the Departments of Medicine and Pediatrics, and as a result the Yale Poliomyelitis Study Unit was founded. It was a logical outgrowth of the setting from which it arose. The Department of Internal Medicine and, to a lesser extent, the Department of Pediatrics at the Yale University School of Medicine in 1930 were both more or less dedicated

to the study of the natural history of infectious disease, as well as to metabolic aspects of disease. There were two major divisions, — infectious and metabolic — in each of the two departments. Francis Blake who headed the infectious disease division of the Department of Medicine had trained Dr. Trask, and had shown him how effective research in the field of infection could be. Dr. Blake and Dr. Trask had carried out studies on experimental measles in monkeys at the Rockefeller Institute in 1920-22. As a result, Dr. Trask was experienced in the handling of these expensive, and at that time, unusual experimental animals which subsequently proved so essential for research on poliomyelitis.

In 1931 there were only about three or four virus laboratories in the United States which could be said to be primarily concerned with investigations on the etiologic agent of poliomyelitis. These were the laboratory headed by Dr. Simon Flexner at the Rockefeller Institute for Medical Research in New York City, the Harvard Infantile Paralysis Commission in Boston under the direction of Dr. Lloyd Aycock, the laboratory in the New York City Department of Health under Dr. W. H. Park, and finally, the Department of Bacteriology at the College of Physicians and Surgeons of New York where Dr. C. W. Jungeblut was working primarily on the endocrine influences which might condition the host and render him more susceptible to poliomyelitis virus. At this same institution Jungeblut's approach had been championed by Dr. George Draper who had become a unique figure in the clinical field of acute poliomyelitis, having been the physician in charge of Mr. Franklin D. Roosevelt's case. In the late 1920's Dr. Draper had been placing much emphasis on host factors and he was inclined to believe that work on poliovirus, in which he had been originally interested, was now outmoded. In any event, in 1931 clinical virology was not listed as a major research fashion; so that it was essentially the introduction of the spirit of clinical investigation and what might be termed clinical epidemiology into the field of poliomyelitis which the Yale group (under Dr. Trask and Dr. Paul) accomplished. Indeed, their opportunity was thrust upon them with the onset of an extensive poliomyelitis epidemic which began in New Haven in June, 1931.

The immediate aim of the workers at Yale was to devise a means of isolating the virus from patients, not only from spinal cords of fatal cases as had been the current practice, but in a bolder plan, from mild abortive or nonparalytic cases as well as severe paralytic ones. In planning these virus studies we had been impressed by a recent experience in the previous year in the nearby town of Middletown, Conn. This community had suffered an autumn epidemic in 1930 of some twenty or more cases.

It had been our privilege to see some of these patients and to note that there had been all grades of severity among them. Many a suspected case had not only no paralysis but little or no stiff neck. Virus studies would have helped materially in making the diagnosis. Thus when a similar epidemic situation appeared in New Haven the following spring, the development of diagnostic techniques for the isolation of virus from clinical cases both severe and mild, particularly from individuals who had the so-called *minor illness,* seemed to be the first thing to try to do.

These plans met with very little support from anyone except Dr. W. H. Park of New York City. Consultations were held with him and also with Dr. Flexner and Dr. Aycock; the latter two strongly advised against this approach as one which was expensive and would not yield valuable results. No positive isolations from the throat had been obtained by anyone in thirteen years and the idea of obtaining poliovirus from the feces had been, according to Dr. Flexner, completely disproved. Monkeys cost from six to eight dollars each. This was regarded as an expensive laboratory animal, and it was deemed unwise for amateurs to spend so much money on this type of research, particularly when it was their initial piece of work in this field and there were many more orthodox things to be done. Nevertheless, it seemed to Dr. Trask and his colleagues that the clinical situation and the recent memory of the 1930 Middletown experience cried out for virus studies on all members of families in which poliomyelitis was suspected. We saw family after family through which there had swept a simultaneous wave of minor illnesses, limited to headache, vomiting, fever and sore throat and occasionally in one member, signs of central nervous system involvement. The usual diagnosis given to these family epidemics was summer grippe. In any event, two strains of poliovirus were isolated from the throats of two cases of "abortive poliomyelitis" that summer and, although for the next three or four years these results were severely challenged by experts, they stood the test of time. During this time the Yale Poliomyeliis Study Unit was supported with considerable diffidence by the Fluid Research Fund of Yale University and a series of small grants from various sources, including the Philadelphia Poliomyelitis Fund, an anonymous donor, the National Research Council, and the Rockefeller Foundation. Starting in 1936 we received the first research grant ever given by the President's Birthday Ball Commission which eventually became the National Foundation for Infantile Paralysis, and finally the National Foundation. The earliest grants amounted to two or three thousand dollars, and by the late 1930's they ran to about twelve thousand dollars, a modest sum indeed compared with grants of today.

But to return to the interests of the Yale Laboratory in the 1930's: it was at this time that the Study Unit set for itself the task of determining whether there was more than one strain of poliomyelitis virus in the United States. A pioneer paper on this subject had already been published in 1931 by Burnet (later Sir Macfarlane) and McNamara from Australia. This paper had been received in the United States with some incredulity and the feeling that there was no telling what kind of poliovirus there might be in such far off places as Australia, but "it can't happen here." One of the most significant papers ever published by members of the Yale Poliomyelitis Study Unit was that designated as number 9 on its list. It appeared in 1933 and clearly described the immunological difference between two strains, as demonstrated by cross-immunization reactions and testing the resistance of monkeys by challenge with homologous and heterologous viruses. This work was carried forward for at least three years and by 1937 it was felt that one could divide the poliomyelitis virus family into at least two and possibly three types. In retrospect, whether we actually had a Type III poliovirus in our collection of strains is not absolutely clear. In any event, the work led in the not too distant future to the recognition that the poliovirus family consists of three members.

In the course of these experiments a number of monkeys which had been supposed to be immunized by inoculating them intracutaneously with formalinized virus, actually developed paralysis. The experimental use of formalinized vaccines as a method of immunizing monkeys brings up the inevitable question as to whether their use in man was contemplated at this early date by the Yale Poliomyelitis Study Unit. The answer is that in the mid-1930's this was a dominant theme in poliomyelitis research and immunization with formalin killed virus was being tried by Dr. Kolmer in Philadelphia and by Dr. Brodie of New York. Their work was unsuccessful, however, and the occurrence of possible vaccine induced cases gave a black eye to the idea of a killed poliovirus vaccine. Had the Unit worked on the subject then, it is probable that much time and effort might have been wasted because the separation of the family of polioviruses into different strains was very crude indeed. Furthermore, at the time infected monkey spinal cord was the only source of virus antigen; the development of a practical vaccine had to await the dawn of the tissue culture era fifteen years later.

The next item of considerable interest was the project undertaken in 1935 by members of the Unit who collected from the literature all examples of studies on serum antibodies in normal inhabitants in different parts of the world. This was the first study on serological epidemiology of any

import, and the results, to our surprise, indicated that the highest rates of poliovirus antibodies occurred in tropical areas where the disease was supposed not to occur. At that time this seemed a paradoxical and unorthodox finding.

In 1937 the Unit was successful in isolating poliovirus from the feces of a child in the New Haven Hospital. Isolation of poliovirus from feces had been achieved once before by a Swedish group headed by Dr. Kling of Stockholm, but their experiments had been challenged by Dr. Flexner. Since 1932 the Yale Poliomyelitis Study Unit had attempted to repeat the work of Dr. Kling and his colleagues but the route of inoculation at that time, that is, into the brain of the monkey, was hazardous because of frequent bacterial contamination and brain abscesses, and no positive results were achieved. Besides, monkeys were limited in quantity and so expensive that it seemed wasteful to pursue this approach. The discovery which led subsequently to widespread use of fecal material in the study of the epidemiology of poliomyelitis came by pure chance. We happened to have a pet monkey in the laboratory named Bosco who was quite a beautiful animal of African origin, a Cercopithecus monkey which we had been conserving for no good scientific purpose at the cost of $1.00 per month. And yet no one had had the heart to use him on an experiment which might cause paralysis or early demise. In October, 1937 a poliomyelitis patient named Skolnick was admitted to the Pediatric Service of the New Haven Hospital and Dr. Alfred Vignec of that service, and Dr. Paul went to the home of this patient where an infant sibling was found to have fever but seemed otherwise normal. Throat washings and a stool specimen, which was conveniently produced at the time of the visit, were collected. It was at the suggestion of Dr. Vignec (now a well-known pediatrician of New York City) that the extract of fecal material from the Skolnick infant was inoculated intraperitoneally into Bosco. Both Dr. Trask and Dr. Paul, and last but not least, the faithful animal caretaker* who was devoted to Bosco, firmly believed that he would safely survive this procedure. But Bosco was paralyzed in all four limbs within a few days of inoculation and, sad as his death was, it opened up a large new field, quickly exploited by the Poliomyelitis Study Unit, even though the idea of isolating a virus from feces had very little appeal at that time to most good contemporary microbiologists. As for poliomyelitis, the accepted theory was that this was a disease in which the agent was spread by way of the respiratory tract. It soon became obvious that it was far easier to find poliovirus in the

* Mr. Alfred Coppola, now working in the Department of Physiology in the same capacity.

feces of patients ill or recently ill with poliomyelitis, than to find the virus in their nasopharyngeal washings, the customary source of virus isolation from living patients. Nevertheless, the devotees of the respiratory transmission theory of the virus still held out.

Following the detection of poliovirus in human feces and the use of stool isolations as an epidemiologic tool, it was a short step to see whether the virus could be demonstrated in sewage. This was soon found to be very easy. The first report on isolation of poliovirus from sewage came from the Yale Laboratory in 1939. Shortly after this a successful effort was made to determine the presence or survival of the virus in flies, particularly the filth flies whose favorite food is feces. These investigations, which came fast upon the heels of one another, gave the Yale Laboratory the reputation that it believed that poliomyelitis was a water-borne disease spread by sewage! It is true that the laboratory had stated early in 1939 that it was logical to regard poliomyelitis as an "intestinal disease" and this pronouncement had been greeted with some dismay on the part of our friends who felt that we had done good work up until then but had finally "gone off the deep end." Actually the attitude which the laboratory soon took was that the presence of poliovirus in sewage provided a useful epidemiological tool, but there was no implication that the agent was spread by sewage. It was pointed out that sewage is full of pathogenic organisms including tubercle bacilli, but this does not mean that sewage is the usual vehicle of transfer of tubercle bacilli.

One of the uses to which the sewage findings were put was to test a theory largely promoted during the 1930's by Dr. Lloyd Aycock of the Harvard Infantile Paralysis Commission. The idea was that there was just as much poliomyelitis infection present in a community during the winter as in the summer, but there was a seasonal lack of resistance on the part of the human host which made him succumb to paralytic poliomyelitis in the summer. This lack of resistance was given the name "autarcesis" and it was produced somehow or other supposedly by hot weather. The discussions about this went on pro and con between Harvard and Yale for several years. It is possible that there was something in Dr. Aycock's argument, but in any event to prove or disprove his theory we went to great lengths. We sampled the sewage of a particular district in New York City each month over a period of six years in order to find out whether poliomyelitis virus was present there regularly month in, month out, during non-epidemic times, as well as during epidemics, as Aycock had predicted. Our findings indicated that poliomyelitis virus, detected by the cumbersome method of monkey inoculation then in use, was present in the raw sewage of New

York City only during the summer and fall and most frequently when there were actual cases of poliomyelitis being reported there.

The presence of poliomyelitis virus in flies is another story. The actual role which they play in the dissemination of the disease has never been established but it seems very probable that in areas that are teeming with flies such as Cairo, Egypt, they do play a role. This evidence is supported by the experiments carried out in the Yale Laboratory in 1943 by Ward, Melnick, and Horstmann, who found that flies in an epidemic area can contaminate food with poliovirus, and when such contaminated food was fed to chimpanzees, poliovirus infection was induced in them. This indicated that flies could at least be mechanical carriers of the virus of poliomyelitis. Arguments as to how important flies are in the usual spread of the disease have been pursued for twenty years without reaching final conclusions. Two projects have been carried out in this laboratory over the years, the first one by Dr. Melnick and others, and a more recent one by Dr. Margrét Gudnadóttir, a fellow from Iceland. The work indicated that flies can maintain the virus for a period of two weeks, and Dr. Gudnadóttir's findings suggest that slight multiplication does take place in the body of the fly. However, there is no evidence that flies play an essential rôle in the transmission of the virus in the manner in which mosquitoes are essential in the transmission of yellow fever or malaria.

The story of the work of the Yale Poliomyelitis Study Unit would be incomplete without emphasis upon the field work carried out during the 1930's. It is difficult today for us to realize how helpless physicians and health officers were in this period in the face of a severe poliomyelitis epidemic. Having issued proclamations in the newspaper, and quarantined the exposed households, there was little else they could do. However, it became fashionable for the health officers to call in a team of epidemiologists or research workers. They did not expect much from these workers but the feeling was that the presence of consultants or of a team of experts might give the public the impression that at least something was being done. This launched the Yale laboratory on a series of clinical epidemiological studies which gave us an opportunity to see the disease first hand in various locations. At first it was our practice to spend a month to six weeks in each epidemic area; in several of these a laboratory was set up complete with facilities for housing monkeys and inoculating them on the spot. Later with the advent of airplane travel, a series of short visits were generally made. Epidemiological studies of various types were carried out starting in New Haven in 1931; Philadelphia in 1932; in Los Angeles in 1934; in Toronto, Canada in 1937; in Charleston, South Carolina in 1939, also

in that same year in Detroit, Michigan, and Buffalo, New York; in 1940, in Indianapolis, Indiana and in Huntington, West Virginia; in 1941 Miami, Florida, Cordova, Alabama, and Winnipeg, Canada, to mention only a few of these expeditions. It is hard to imagine the excitement and the misguided publicity which attended the arrival of the research workers in an epidemic area in the 1930's. In the long run, however, the time and effort spent in these endeavors cannot be considered wasted. Occasionally there was bitterness on the part of local research workers, but more often these ventures spelled the beginning of fine pieces of collaboration.

In the 1940's there came a new stage of investigation and the first half of this decade was taken up with problems in connection with World War II. Early in 1941 with the formation of the Army Epidemiology Board, a Commission on Neurotropic Virus Diseases was formed as a part of this Board, and the directorship was established in the Yale Laboratory (Dr. Paul). The mission with which the Commission was charged was to study the arthropod-borne virus diseases and poliomyelitis in members of the U.S. Armed Forces. Early in the war it was felt that poliomyelitis was certainly not a military disease, but this was soon to be disproved and several outbreaks in Army camps, such as Fort Brooke in San Antonio, Fort McClellan in Alabama, required the assistance of members of this commission. Prominent among its members were Dr. Trask, Dr. Robert Ward and, as assistant, Dr. Melnick. In May of 1942 Dr. Trask, the leader of the Yale Poliomyelitis Study Unit, died at the age of 52 while working in an Army camp in Illinois. It was a very severe blow to his colleagues and to the Commission and to the cause of poliomyelitis. Through the influence of the National Foundation for Infantile Paralysis, Mr. Basil O'Connor, its President, made special provision that a 10,000 ton Liberty Ship built and christened in the shipyards of Baltimore, Maryland in May 1944, be given the name, The James D. Trask, in memory of this singularly able scientist and clinician, in recognition of his contribution to the study of poliomyelitis.

In 1943 the forces of the Commission were divided with Dr. Paul heading a small group (including Major Albert Sabin and Lt. Col. Cornelius Phillip) known as the Virus Commission of the Army Epidemiological Board, which was dispatched to North Africa to study problems in connection with infectious hepatitis, sandfly fever, and poliomyelitis. The team that remained at home consisted of Drs. Ward, Melnick, and Horstmann who were very active in clinical investigative work carried out during the epidemic of poliomyelitis which visited New Haven during that summer of 1943 and about which a number of important papers were published.

The story of poliomyelitis in North Africa is worth repeating. During the winter of 1942-43 there had been received at the Rockefeller Institute several specimens of central nervous system tissue originally obtained from fatal cases of myelitis and encephalitis occurring in British soldiers serving in the Middle East Forces. This was the period of World War II when the Middle East was the most critical area. Rommel had come within a short distance of Cairo. The German plan was to take Stalingrad and eventually as part of a pincers movement to work south to join forces with the Afrika Korps. It was at the Battle of El Alamein in early October of 1942 that Montgomery and the British Eighth Army had upset Rommel's plans, and these plans were to be further upset early in November with the landing of U.S. troops in North Africa, in French Morocco and Algeria. During this period British troops in the Middle East and the Mediterranean area had been severely plagued with epidemics of viral hepatitis. They also had begun to have sporadic cases of a serious disease of the central nervous system, often with ascending paralysis, and in many instances, with a fatal outcome. Was this poliomyelitis? Certainly encephalitis or poliomyelitis had not been the experience of previous British armies in Egypt. Furthermore, poliomyelitis was said to be rare among the civilian population of Egypt and other areas of the Middle East. In Egypt not more than five or six civilian cases per year had been reported in the late 1930's among the native population of 16,000,000 people!

It was but a matter of a few weeks to determine that the central nervous system tissue sent to this country and examined first at the Rockefeller Institute contained poliomyelitis virus. This finding was one of the reasons that our Commission was sent over to North Africa to investigate the situation. On arrival in Cairo, the laboratory was set up in conjunction with the 38th U.S. General Hospital (the Jefferson Medical School Hospital), and work was commenced at once on the cases of suspected poliomyelitis in U.S. troops which began to come in some numbers. Medical officers of the U.S. Army, especially the neurological consultants, were very skeptical about the clinical diagnosis of these cases. The clinical picture did not conform to their ideas of adult poliomyelitis, but the repeated isolation of strains of poliovirus from such cases was eventually convincing. The virus isolation work was carried out in monkeys which had to be obtained locally. Some of the members of the Commission (J.R.P.) had to visit Ethiopia in order to trap their own animals and bring them back to the laboratory in Cairo.

It was in this setting that an important epidemiological idea began to come into being. It grew out of the observation that adult poliomyelitis

could exist and even thrive among visitors (i.e. susceptible immigrants) in an area where only *infantile paralysis* had been previously recognized; where even that was thought to be very uncommon; and where adult poliomyelitis among the native inhabitants was unknown. This indicated that young men of military age coming from countries with high standards of hygiene in which epidemic twentieth century poliomyelitis was plentiful enough, — such young men were highly susceptible when introduced into regions where sanitary arrangements were about as primative as could be imagined. Thus on exposure to populations in which poliovirus infection was probably endemic, they became infected readily, and as a result poliomyelitis began to crop up in the soldiers from England and from the United States. It seemed that poliomyelitis had become a military disease during the generation which separated World War I from World War II. During this period, in many of the more "developed" parts of the world it had been noticed that the age at which the disease was occurring had been pushed up a decade or more. In other words, people were acquiring poliovirus infections later in life than they had before, and as a result many arrived at military age without having had the benefit of previous inapparent immunizing infections. This was but one of the many examples of the changing pattern of poliomyelitis which subsequently became evident.

During this early part of the 1940's the Yale laboratory continued to send out teams to carry on field work in epidemic areas. Notable among these expeditions were those to Hickory, North Carolina in 1944, to Western New York State in the same year; to Patterson, New Jersey, and to Rockford, Illinois in 1945.

By the end of the 1940's some very important discoveries had been made. Previously Dr. Charles Armstrong of the National Institutes of Health had indicated that one strain of type II poliovirus would infect mice, an enormous boon to laboratory workers. Later, working under the National Foundation for Infantile Paralysis, a team of investigators in several laboratories had separated the polomyelitis virus family quite definitely into a group of three, types I, II, and III. This brought considerable order out of great chaos. And finally, in 1948 came the monumental discovery by Drs. Enders, Weller, and Robbins that the virus of poliomyelitis could be grown in tissue culture. The importance of this was not fully appreciated at first, but it was one of the greatest milestones in the history of the disease.

During the latter half of the 1940's the Yale Poliomyelitis Study Unit busied itself in large measure with developing the concept that poliomyelitis was a disease in which the epidemiologic picture differed in different parts of the world. Further studies on the distribution in troops in World

War II, particularly in Japan, brought forth some interesting findings indicating that the rate at which soldiers acquired the disease in the Philippines, China, Burma, and India was ten times greater than the rate at which the disease was acquired by troops of similar age who remained at home.

Very shortly after these observations were made, a dramatic example of the behavior of poliomyelitis in highly susceptible populations in the arctic occurred in the form of an epidemic among Canadian Eskimos in 1948-49. There, in the fall of 1948, the disease was first introduced into settlements along the west coast of Hudson Bay. It spread rapidly northward where the epidemic eventually involved almost twenty per cent of the entire population with paralytic disease. All age groups were attacked, the highest rates of paralysis occurring in adults, and the lowest in infants! Stimulated by this event, the Yale Poliomyelitis Study Unit (J.R.P.) made an expedition in 1949 to Point Barrow on the north coast of Alaska, where in connection with the Arctic Research Laboratory an attempt was made to see whether the non-immune character of the Eskimos living in that part of the world could be demonstrated by serological means. Available tests for carrying out a study in serological epidemiology at the time were crude but, nevertheless, the study did prove of considerable value. It was found that among the members of the Point Barrow community virtually none under the age of twenty possessed antibodies to type II poliovirus, whereas about seventy per cent above twenty years did possess such antibodies. The ages of persons with type I antibodies was 35 and over, and with type III, 45 and over. This was a pilot study in serological epidemiology and opened a whole new field. It appeared that from the determination of the pattern of antibody distribution on an age specific basis, it was possible to retrace the history of an infection in an area where little or no accurate vital statistics had ever existed.

As a quick corollary to the Alaskan study, the next expedition which the Study Unit organized (and J.R.P. carried out) was to a part of the world which was selected because it is as completely opposite to barren arctic wastes as could be imagined, namely to a crowded Egyptian village in the outskirts of Cairo. It was felt that if the population of Cairo was sufficiently immune so that no adults ever acquired clinical poliomyelitis, it should be possible to document this fact in their antibody patterns. Such was indeed found to be the case: by the time they reached 3 or 4 years of age virtually all Egyptian children had antibodies to the three types of poliovirus. As an important by-product of this study, an alert graduate student working in the Section of Preventive Medicine found that

in some of the bloods which were being tested for antibody content there was a virus present, and this agent was quickly identified with the help of Dr. Melnick as West Nile Fever virus. This virus has subsequently been shown to be the cause of a great deal of mild febrile illness in Egypt and in Israel. Although it had been discovered ten years earlier in Central Africa, the agent had never before been found so far north in Africa, and its importance as a producer of illness had gone unappreciated for many years. Credit for this discovery is due to Miss VoHammie Barnett who died a few months after making this remarkable observation.

Subsequently, serological surveys were carried out by the Yale group in other populations. These included the populations on the island of Tahiti, in Morocco, North Africa, in North Carolina, and later in Guatemala, Central America. During this period there was a sudden increase in the use of the antibody survey as a method of determining the immune status of given populations throughout the world.

Also in the 1940's, came the discovery of the Coxsackie group of viruses by Dr. Gilbert Dalldorf and Miss Grace Sickles. Dr. Melnick immediately became interested in work with these agents. Together with Dr. Edward C. Curnen, strains were isolated from cases of aseptic meningitis. Subsequently, Dr. Melnick and his collaborators were in the forefront in classifying this large family of agents, characterizing their physical properties, and studying their pathology, serology, and epidemiology.

We come now to a new decade and a new era in the history of poliomyelitis, for the 1950's marked the beginning of the era of the development of active immunization. The advent of the tissue culture method and the discovery that poliovirus produces a cytopathogenic effect on tissue culture cells by which its presence can be detected, enormously facilitated research work in this field. It was possible not only to isolate poliovirus readily and demonstrate antibodies, but means were now available for preparing a vaccine. On this basis, the work of Dr. Salk began early in 1952 and progressed successfully to the production of the vaccine for general use in April 1955. We have been asked many times why the Yale Laboratory having had such a fine background and having begun work on this very subject in 1936, never really pursued it to a satisfactory conclusion. Actually, the first formalinized killed vaccine antigens were administered by the Yale Poliomyelitis Study Unit in 1931 when Dr. Trask and Dr. Paul both were inoculated with small doses of formalinized vaccine to see if they could boost their own antibodies and quantitatively demonstrate this in the clumsy neutralization test in monkeys. It was not, obviously, a successful procedure. Killed vaccines had a bad name in the mid and late 1930's

and the antibody tests were so poor that a book was written discrediting them almost completely.

The next efforts of the Unit to immunize man were made in 1946. A rather unfortunate choice was made at this time in that heat killed mouse brain virus was selected as the antigen for a vaccine. The decision to use heat inactivation rather than formalin was based partly on our earlier immunization experiments in monkeys carried out from 1935 to 1938; in several instances the formalin had failed to kill the poliomyelitis virus and we felt insecure about its adequacy. Obviously, we did not know how to use it. Our first vaccination trial in man was an effort to boost antibodies by inoculating the heat killed antigen. This work was carried out in Miami, Florida in 1946 by Dr. Horstmann. The method of measuring antibody rises by intracerebral neutralization tests in mice left a good deal to be desired. The results were not particularly successful; had they been a little more promising, we would have pushed forward at that time with all possible speed.

During the period 1946-49 we continued experiments on the use of heat killed antigens which were used not only in attempting to develop a skin test, but in an effort to immunize man. The work was carried out at the Southbury Training School, Southbury, Connecticut. At first we used as antigen the YSK (type II poliovirus) propagated in cotton rats, and heat inactivated at 70° for thirty minutes. Eventually, it was found that by concentrating the antigen one hundred times by ulracentrifugation, and by reducing the heat of inactivation from 70° to 60° C. the antigenicity was improved. In fact three of fourteen individuals tested showed rises in antibody after inoculation of this preparation. Unfortunately heat causes deterioration of the antigen to a far greater degree than does formalin; if only the result had been a little better than 20% we would have gone forward on this lead. At the same time we began to work with oral immunization with heat killed material and shortly thereafter Dr. Melnick began to work on the attenuation of poliovirus strains which might be used in a vaccine. In all these experiments the results were a little under the level which would have caused us to pursue them with greater vigor.

In 1951, Dr. Horstmann again began work on the question of viremia in poliomyelitis. This had been studied in 1943, but at that time virus had been isolated from the blood of only 1 of 111 patients with the disease, and the conclusion was reached that viremia was rare and unimportant in the pathogenesis of poliomyelitis. In 1951 and 1952, however, we found that orally infected monkeys and chimpanzees regularly had virus in the blood. However, viremia was detected only in the incubation period and

not after signs of the disease appeared. In the summer of 1952 Dr. Horstmann and Dr. Robert McCollum demonstrated viremia in human infections, in blood specimens collected from family contacts of paralytic cases, from those with asymptomatic infections, and from children with the "minor illness", the study having been carried out during an epidemic in Ohio. At about the same time in 1952, Dr. Bodian and his colleagues at Johns Hopkins also demonstrated poliovirus in human blood.

During the early 1950's the Unit pursued its work in serological epidemiology and under Dr. Melnick, many studies were carried out, developing and modifying the new tissue culture techniques. Mr. John Riordan, Dr. Edward Opton, and later Dr. Edith Hsiung and Dr. Francis Black participated extensively in this work. In 1955 the laboratory began to plan and carry out a series of field trials with the various oral poliovirus vaccines. This latter work has more or less dominated the Yale Poliomyelitis Study Unit program during the last several years. A series of live poliovirus vaccine trials using Sabin's strains was carried out at the Southbury Training School, by Drs. Paul, Horstmann, Niederman, and Deutsch in cooperation with Dr. Herman Yannet, the medical director, of the institution. Another trial was carried out among the Yaqui Indians in Southern Arizona, and still another, using the Lederle vaccine, in a selected village population in Costa Rica. All of these trials were set up in such a way as to yield information about the epidemiology and pathogenesis of poliomyelitis, as well as about the safety and efficacy of live poliovirus vaccines. The concept of local tissue immunity (in addition to humoral immunity) as a major defense mechanism was advanced as a result of the observations made. The responsibility of gathering information on the safety and efficacy of the vaccine has been a concern of the Unit particularly because the Director (Dr. Paul) has been a member of the Committee on Live Poliovirus Vaccine of the National Institutes of Health in which important decisions concerning the use of oral vaccine have been considered over the past three years.

It should be mentioned that during the past seven years the Yale Poliomyelitis Study Unit has acted as the World Health Organization's Laboratory of Poliomyelitis for the Americas. This is a Regional Laboratory which deals with certain problems of a reasearch and service nature concerning this disease in the Latin American countries.

This report would not be complete without mentioning the names of those fellows and younger members of the Section who have made a career for themselves in the poliomyelitis field. The following in particular might be mentioned at this time: Dr. Herbert A. Wenner, Research Professor of

Pediatrics at the University of Kansas; Dr. Robert Ward, Professor of Pediatrics at the University of Southern California Medical School; Dr. Herdis von Magnus, Chief of the Piliomyelitis Department of the Statens Seruminstitut of Copenhagen, Denmark; Dr. Sven Gard, Professor of Virology, Karolinska Institute Stockholm, Sweden; Dr. Edward C. Curnen, Professor of Pediatrics at the College of Physicians and Surgeons, Columbia University, New York; and Dr. G. Contreras of Santiago, Chile, Dr. Natan Goldblum of Israel, Dr. Ramos Alvarez of Mexico City, and last but far from least, Dr. Joseph L. Melnick, Professor of Virology and Epidemiology at Baylor University in Houston, Texas.

Since the establishment of the unit a series of more than 325 papers on poliomyelitis have been published and a review of these titles has fairly well covered the story of the disease during this thirty year period.

STUDIES ON VIRAL HEPATITIS

Yale's contribution to the study of the etiology of infectious hepatitis deserves some mention. One of the first tasks of the various commissions of the Army Epidemiological Board, of which Francis G. Blake was appointed president early in 1941, was to investigate the outbreak of hepatitis which occurred in the Army as a result of the use of yellow fever vaccine among troops in 1942. Until 1944-45 there was no appreciation of the fact that there are two forms of hepatitis, the so-called *infectious* hepatitis and *serum* hepatitis. Whether the difference between these two forms is due to their being caused by two completely distinct viruses or by two varieties of the same virus is still a moot point; but it is sufficient to state that there are differences between the agents, and their modes of transmission differ, however indistinguishable clinically the two diseases may be at times.

In 1942 most physicians in the United States, and for that matter in the greater part of the world, recognized an epidemic form of mild jaundice, distinct from Weil's disease. It was an ill-defined entity, however, rather uncommon, and felt to be probably different from the common sporadic disease known as catarrhal jaundice. The spirochetal jaundice of Weil's disease was recognized as a diffuse hepatitis, whereas catarrhal jaundice was considered to be an obstructive jaundice due to primary inflammation of the bile ducts, and quite different in its pathology, prognosis, and clinical picture. This view had been held for a long time despite the fact that there was good clinical and epidemiologic evidence in the literature to indicate that it was incorrect.

Dr. George Blumer of this School had been among the first in this country to uphold the idea that catarrhal jaundice was the sporadic form of

infectious hepatitis. His views were published in the Journal of the American Medical Association in 1923, shortly after an epidemic had been described among Yale students by Dr. Ira Hiscock among others. However, Dr. Blumer's view had been anticipated in a report by Cockayne in England published in 1912, giving an historical review with the comprehensive title, "Catarrhal Jaundice, Sporadic and Epidemic, and Its Relation to Acute Yellow Atrophy of the Liver." Cockayne's views and also those of Blumer received very little attention for many years. Catarrhal jaundice, it was insisted, was a catarrh of the biliary tract in which mucus plugs played a role. So strong was this view that one school championed the idea that a rational, direct, and effective method of therapy was the removal of this plug of inspissated mucus "corking" up the ampulla of Vater, by the introduction of magnesium sulfate into the duodenum. The great pathologist Virchow had described this plug and accounts of its supposed importance had been carried through successive editions of many textbooks (including the influential Osler's text) for almost two generations. Eppenger in Vienna was the first to suggest that the pathogenesis of catarrhal jaundice was really associated with hepatocellular necrosis. His paper was published in 1922. Eight years later Eppenger's views were upheld by Dr. Aronld Rich who, after studying the records of 11,500 autopsies performed at the Johns Hopkins Hospital during 40 years, reported that there was not a single case among them in which the diagnosis of "catarrhal jaundice" could be regarded as accurate. Thus, it seems that the concept of the pathogenesis of the clinical entity known as catarrhal jaundice had gone beyond the facts.

Outbreaks of serum hepatitis on the other hand, dated back to about 1883, and were observed following vaccination for smallpox, the use of arsphenamine in the treatment of syphilis, of insulin in diabetic clinics and in other situations in which the parenteral administration of drugs was used. Outstanding in the history of serum hepatitis were the cases which occurred during World War II following vaccination against yellow fever. At this time the virus used in yellow fever vaccine was grown in tissue cultures containing human serum in the media. In spite of this information, however, the epidemic of serum hepatitis fell upon the unsuspecting American Army like the bombshells falling on Pearl Harbor. As a result, many investigators in this country took upon themselves the study of a comparison between epidemic hepatitis and serum hepatitis. There seemed to be a difference: — one had a long incubation period ranging from 60 to 150 days, the other a shorter one from 15 to 40 days.

A great advance in the study of infectious hepatitis took place when the disease was first produced experimentally in man. In 1942, Voegt, in Germany, reported the transmission of hepatitis to volunteers by feeding them duodenal fluid or blood obtained from patients in the acute phase of the disease. Unfortunately, published accounts of his experiments are quite inadequate. In 1944, MacCallum and Bradley in England, at the Jaundice Center of the British Medical Research Council, obtained some highly significant experimental results. Their report, in the form of a letter published in the *Lancet* on 12 August 1944, described success in transmitting viral hepatitis to arthritic patients. Various routes of transmission had been used and the infective material included feces given by mouth. Independently, members of the Neurotropic Virus Commission here at Yale working largely under the direction of Major W. Paul Havens were successful in producing the disease in volunteers of Civilian Public Service Units, composed of conscientious objectors who were quartered in New Haven. These men were fed fecal specimens obtained from soldiers in the Mediterranean area during the epidemic of 1943. The experiments were begun in June and July of 1944, and the patients were housed on the infectious disease wards of the New Haven Hospital. The first definite experimental cases occurred in July and a report of this work was presented at the first meeting of the Hepatitis Study Group, held at the Rockefeller Institute for Medical Research in New York on 12 July 1944. It was published in November 1944. It can be fairly stated that this represented the first published successful demonstration in the United States of the enteric-oral transmission of infectious hepatitis.

Other aspects of the disease which were investigated in New Haven included the determination of the times at which virus could be found in the blood and in feces, both before and after the onset of jaundice; and an excellent study of the blood picture. Cross protection studies in volunteers indicated a lack of cross immunity between serum and infectious hepatitis. From this work, and from studies carried out elsewhere, a differentiation between the two hepatitis viruses was established, and the present concept of the two diseases emerged."

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THOMAS FRANCIS, JR.*

Department of Epidemiology, University of Michigan School of Public Health, Ann Arbor

PROBLEMS OF ACUTE RESPIRATORY DISEASE†

FOREWORD

In the period of John Paul's lifetime in medicine, great changes have occurred in the face and philosophy of infectious disease. His work has contributed greatly to those changes. Being of sound and inquisitive mind, itchy foot, and unlimited resources, he has moved through many intellectual and geographic areas in unending pursuit of pathogenic facts and inferences. When he went to Yale in 1928 he joined the stimulating company of Blake and Trask in an association which I, too, had enjoyed. This team of Paul and Trask produced a remarkable combination of bold and meticulous research, bringing clinical, pathological, and microbiological competence to epidemiological studies of poliomyelitis. As Director of the Commission on Neurotropic Virus Diseases for fifteen years, he brought together a splendid group of investigators whose contributions have been legendary. And, of course, studies of hepatitis grew under his art and persuasion.

It has been a rare privilege to have had close and continued association with John Paul over these many years; to watch the determined approach to difficult problems with courageous and imaginative skill. But there have also been many periods to enjoy in a relaxed manner, social, and cultural interests he so expertly developed. It has been a great life whose lively facets will continue to glisten and to gain luster long after the official academic curtain has fallen.

Many forget that John Paul was earlier a student of respiratory disease and its familial distribution. When I first knew him he was, like so many of the modern virologists, a pneumococcus expert. It seems quite fitting, then, to present as a token of respect for his versatile achievements and of personal affection, some remarks on the Problems of Acute Respiratory Disease.

* * *

The oft-repeated statement that the infectious diseases are conquered seems ridiculous when one considers the number of cases and com-

*Chairman, Department of Epidemiology.

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plexity of acute respiratory disease. The U.S. National Health Survey reports that in the year July 1957-June 1958[1] among the noninstitutionalized population, 36.5 percent of work-loss days, 45 percent of bed-disability days, and 67 percent of school-loss days were caused by acute respiratory disease. It constituted 60 to 66 percent of all acute conditions in every age group. In comparison with 764 million days of bed-disability for all chronic conditions, were 593 million days caused by acute respiratory disease. Although 1957-58 was an epidemic influenza year, data for 1958-59[2] and 1959-60[3] still emphasize the great segment of illness and disability created by acute respiratory disease as measured in total incidence, incidence rates, or bed days.

The burden is especially heavy during the developmental period of life. Since we have a "younging" population, with an estimated 40 percent under 20 years of age by 1970, it is clear that the bulk of respiratory disease looms progressively larger. The vigorous, productive years are also repeatedly disrupted. The work-loss from acute respiratory disease in 1957-58 was 219 million days; in 1958-59, 190 million, in comparison with 226 million work days lost from circulatory, digestive, and arthritic diseases and impairments from injury or other origin. These urchins of frequent, repetitive respiratory illnesses clearly take a formidable toll. On the basis of general experience and comparison of mortality rates from respiratory disease, it seems evident that the situation is no less prominent in other nations and regions where other infections and nutritional inadequacies present additional serious loads.

The size of the problem is compounded by the multiplicity and heterogeneity of the etiological agents: bacterial, viral, fungal, and others. This complexity was early postulated on epidemiologic and immunological grounds, but the actual identification of numerous serologic types among pneumococci and other bacteria prepared investigators for a similar pattern among the respiratory viruses. It developed rapidly with recognition of the influenza viruses, the adenoviruses, the hemadsorption viruses, some enteroviruses, some not yet clearly classified, reoviruses, or a group which the British Salisbury team suggests may be sufficiently different and characteristic to classify separately.

The response to infection varies widely from the inapparent, the afebrile with respiratory symptoms, and the mild febrile illness to severe and pneumonic disease. Variation occurs also with age, season, experience, strain, or special conditions such as institutional or military life. Therefore, a great bulk of the illness is more an epidemiologic syndrome than distinct clinical syndromes readily associated with specific etiological agents. The

popular diagnosis of "a virus" is thus fairly precise. Nevertheless, some of the agents tend to incite a certain set of clinical signs with greater regularity than others so that within these limits of clarity an etiological relationship may be suspected.

INFLUENZA VIRUS STRAINS

Generally, clinical influenza is recognized in epidemics occurring at relatively short intervals. They are most frequently associated with type A virus. For a decade or so strains of a closely related antigenic character spread about the world in large and small prevalences so as to give a high level of immunity. A popular thesis held that these recurrences were the result of a directional alteration by which the successive strains were progressively discarding the characteristic antigen of the previous year and picking up entirely new constitutive antigens. The evidence does not appear in keeping with that idea.

Rather, the strains of a period appear to comprise a group or family with close similarities, but varying quantitatively in a random fashion about a central pattern. This hypothesis, which seems in accord with the cumulative information, is that the virus comprises a large number of demonstrable antigenic components which may, by rearrangement, develop phenotypic alterations about the prototype. They do not represent progressive recessions from the initial strain becoming increasingly remote with the passage of time.[4]

Nevertheless, at intervals of 10 years or so, we have witnessed the appearance of major variants whose dominant antigen is relatively new to most of the human population. They are the Asian variant of type A (1957), the A′ (1947), the initial A (PR8-WS 1933-34), and the swine influenza virus. That they are not entirely novel is indicated by the reduced incidence of the disease in the older segments of the population, by the demonstration of antibody in serum of the adult population before the recognized appearance of the strain, as in 1947 and 1957; and by the appearance of antibody to swine virus, in convalescent children for example, at a time when the virus as such is not demonstratably prevalent. Each of these features tends to emphasize the expression of common antigens existent in various strains.[5]

The pattern of antibody response appears in infancy and childhood to be largely directed against the dominant antigen of the prevalent virus family. With successive exposure that antibody is steadily reinforced by the stimulus of similar, though secondary, antigens of infecting strains so that it persists at high levels and constitutes the antibody characteristic of that

cohort throughout life. In the meantime, antibody to other constituent antigens is also acquired and a broad resistance, less influenced by strain characteristics, is thus developed.[6]

What are the mechanisms by which these major sports come into general circulation? It has been suggested that they arise in many parts of the world simultaneously rather than spreading from area to area by transfer. It seems quite unlikely that, with the numerous arrangements possible, one combination would erupt spontaneously in such a diffuse manner. The evidence of spread from place to place has been so clearly charted repeatedly, especially in 1957, that little doubt remains of this fact. Moreover, the antibody pattern of the population in widely separate regions is much the same in a given period of time, indicating that most strains do become disseminated throughout the world whether associated with recognized large pandemics or not.

What are the other possibilities? Dr. Richard E. Shope has long been interested in the possibility that a masked form of virus persists and can be activated by varied, provoking stresses.[7,8] Our knowledge would require that there be a malleable viral nucleus which can be activiated to yield various antigenic patterns rather than a relighting of the same old virus. I do not think this too difficult to visualize as a biological phenomenon, and it could occur in man or in other species.

But there is an alternative proposal: The virus maintains an essentially fixed character in another host and will reenter the human population when conditions are favorable. The swine virus of the United States is an example. The same idea has been expressed by Mulder[9] about the 1957 Asian strain—that it has been resident among swine in Asia, maintaining its antigenic composition over many years, before reappearing in the human population. We agree that a virus with a similar dominant antigen was prevalent in the 1889-90 period and that the antibodies to this antigen found in older people before 1957 refer to their childhood experience with a similar strain. But if animal sources of fixed strains are the common basis for human epidemic or pandemic recurrences, then certainly this reservoir has yet to be demonstrated. The recent identification of strains from horses and ducks has not led to serological recognition of their dissemination in man; nor have the animal surveys since 1957 led to any significant evidence of distribution of the 1957 virus in various animal species. Zhdanov[10] has suggested that the animal strains are old human strains which have become so established in those hosts as to represent errant dead end kids. In the gospel of St. Matthew it tells of the casting of devils into swine; that was probably influenza. But we are dealing in these respects

with only secular revelations, and the entire subject must be kept under critical scrutiny.

On the other hand animal diseases, even respiratory infections, are not frequently transmitted from man to man. (Perhaps pneumonic plague is an exception.) The rapid variation in influenza stains suggests *rapid* transfer. The widespread rapid distribution of the numerous influenza mutants emphasizes the active role of human agencies. But even sharp antigenic variation is not necessarily associated with increased virulence or epidemic dispersibility. In fact, these two characteristics are to a large extent independent variables. We have recorded the shift with years in the age distribution of antibody in the human population to the various type A families and believe that this altering base of immunity is an important factor in selection and dissemination of the major influenza variants. Since the breadth of antibody is dependent upon experience with strains of varied composition, inexperienced children exhibit the highest incidence of infection by any or all strains, and also most limited reflection of viral antigens. With the passage of time antibody to succeeding dominants is acquired, but large gaps develop in the immunity of the community to viral antigens which prevailed when the older segments of the population were young. These gaps invite a rearrangement of viral antigens to enter the population and provide the opportunity for rapid passage and development of epidemic potency. Thus, a resurgence of older or suppressed antigens occurs to complete a cycle of antigenic repression and renewal.

Such a variant may arise in any region, and if transfer to other susceptibles is furthered by crowded conditions, it may increase its epidemic potential, possibly its virulence, and proceed on its adventurous path. I believe that the 1957 strains are notable support for this hypothesis and that a swinelike strain can be expected one of these days. That the antigens for this remodeling are still available in current strains has been already indicated in data from the virus laboratory at the University of Michigan.[23] Maassab obtained indications that fresh glacial acetic extracts of Asian strain induced antibody in mice which cross reacted with PR8 and swine strains, while whole virus did not. Berlin and Minuse both showed that hyperimmunization with the Asian strain gave cross protection against the PR301 strain of 1954. Davenport and Hennessy have recently shown that immunization of rabbits with ether-purified HA fractions of the PR8 strain induced cross titers to FM-1 and swine strains while the intact virus did not. Mulder[9], and Lief and Henle[18] have brought additional evidence of the common antigens in Asian virus and earlier A strains. It is on this basis

that our concepts of control and prevention by vaccination have been developed.

OTHER RESPIRATORY AGENTS

While influenza represents the most consistent and prominent cause of general epidemics it is also apparent that the large body of day-by-day respiratory infections is of other etiology. The institutional studies of Huebner, Bell, Chanock, and co-workers[13] have sharply emphasized the steady smog of respiratory infection which constitutes the child's garden of viruses. Again, they are commonly clinical nondescripts, epidemiologically obscure, and even identification of a viral or bacterial agent is at times of doubtful etiological significance. Nevertheless, certain patterns have emerged in distinct form.

One of the most interesting variations is the behavior of the adenoviruses. Originally recognized as unsuspected residents of tonsillar tissue, they have become increasingly notable as agents of sustained epidemics in military recruits, especially types 4 and 7.[14] They may account for as much as 80 percent of the febrile respiratory illness in those populations but, surprisingly, are ordinarily of small moment among college populations of similar age. It is possible that in recruit populations a minor infection is accentuated by the concentration and constant renewal of susceptibles under unusual physiological stress to a position of exaggerated clinical severity. Theoretically, one would like to postulate the provocation of a latent virus under these conditions. Serologic evidence indicates, however, that recruits do not arrive with a latent infection but acquire it during the period of intensive basic training.

Seasoned troops remain essentially immune. It appears, then, that the viruses remain in the camp, reaching steadily for the new arrivals, and are therefore a place infection. Among children Bell and co-workers[15] have noted the apparent reactivation or reappearance of virus in the same individuals at sporadic intervaals for months after the initial infection. Moreover, there are indications that the reappearance is more common in the winter than in the summer months. This type of intermittent excretion may well be the source for continued infection among recruits.

Certain aspects of the human situation are appearing also among adenovirus infections being recognized in calves, pigs, and chickens. There are many intriguing possibilities in this finding. But the hopeful fact is that inactive adenovirus vaccines containing the commonly involved types have been repeatedly shown to be highly effective. Certain studies have demon-

strated that combined influenza and adenovirus vaccine is highly effective against both sets of viral respiratory disease.

The types prevalent among children and civilian adolescents are ordinarily different from the military, and often illness or antibodies develop without clear evidence of onset. It seems likely, as with pneumococci or streptococci, that different types form groups of different significance.

I shall not pursue further the etiogolical analysis of acute viral respiratory disease except to emphasize again the complexity and multiplicity of the agents. It is important to recall however, that bacterial agents continue to play an important role in acute respiratory disease, especially with respect to mortality. Influenza and pneumonia, mostly bacterial, are still the great killers of infants and a progressive threat to those over 45 years old. More than 350,000 cases of "streptococcal sore throat" were reported in the United States in 1960. In Michigan 9,300 cases of scarlet fever and 1,700 cases of rheumatic fever were reported in 1960. The roles of hemophilus influenza and pneumococcus are still of major consequence.

PREVENTIVE MEASURES

With this array of participating agents, what control or preventive measures can be visualized?

1. I believe that there still remains a hope for hygienic barriers compatible with normal social activity. Our inadequacy cannot seem much greater than that of the fecal hygienist of a century or so ago. If essential extrahuman reserviors exist, as with tuberculosis or brucellosis, efforts can be directed toward their suppression.

2. The progressive knowledge of antigens, antibodies, and immunity continues to direct attention to specific preventive immunization. Vaccination against epidemic influenza can be effective if properly used, with preparations combining the proper types and strains. The evidence exists as well for certain adenoviruses. Is it, then, necessary to assume that each and every one of the agents must be used in vaccines to give the total effect required? I do not know. It may be, as I think has been the case in enterovirus outbreaks, that the suppression of certain agents paves the way for a buildup of others.

On the other hand, rapidly increasing knowledge points to the sameness in structure and basic pathogenesis among many of these agents. It has been easy to visualize and demonstrate the feasibility of compounding vaccines with numerous components and various agents. This practice is enhanced by the use of adjuvants so that small amounts of purified antigens can be immunologically effective and multiple antigens can be employed. They can

be more potent as primary immunizers and boosters, and their effects can be sustained at higher levels for longer periods. At the moment this is the best outlook for specific immunization. But advances in the knowledge of virus transformation, recombination, and genetic determinants may provide simpler methods for compiling a basic immunizing unit of many potentials from essential chemical components.

3. Some form of enhanced general resistance of high efficiency has been considered for a long time. There is little doubt in my mind that much of the decline in mortality from certain diseases, such as scarlet fever, before antibiotics was the result of increased protection in childhood with consequent effects on the vigor of physiological resistance. One day the many splinters may be molded into a functional system which can be modified intelligently. Complement, opsonins, phagocytosis, clonal selection of cells or their stimulation, and properdin, all indicate the possibilities. New agents and new adaptation might well break through these basic mechanisms. A broad refractoriness such as occurs in cellular repair, or the recently discovered interferon, point in other directions. Interference with infection or epidemics (Sydenham) may be developed with attenuated viruses. But I believe that, of itself, this approach provides only a first line of defense.

4. Finally, in my opinion, the most likely resources at present are related to chemoprophylaxis and therapy. The data from our laboratory alone have shown the feasibility of protecting against experimental disease, such as paralytic poliomyelitis in monkeys. The rapidly advancing knowledge at the molecular, cellular, and organic levels of the mechanisms of viral development and infection can be expected to reveal common factors or reactions susceptible to chemical alteration. Consequently, the hope is to override the restrictions of multiple immunological types just as is true in chemoprophylactic control of pneumococci, streptococci, and others, in which the prophylactic effects have been clearly demonstrated by reduction of mortality and disease incidence. Our studies[15–17] are clearly pointing out the way in which drugs may differentiate the significance of RNA and DNA in viral development and exert specific influences on the RNA and DNA viruses.

The problem is still great; the need for new ideas, persistent optimism, and bold undertakings is even greater.

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CHRISTOPHER H. ANDREWES*

Common Cold Research Unit, Salisbury, Wiltshire, England

THE TAXONOMIC POSITION OF COMMON COLD VIRUSES AND SOME OTHERS

During recent years workers in several countries have isolated a number of "new" viruses from upper respiratory infections. For some of these viruses claims have been made that they cause common colds. Before accepting such claims it is well to be sure of two things: i) that the viruses can be recovered with reasonable frequency from typical common colds in adults; and ii) that they will reproduce typical common colds in volunteers. The second criterion is more easily fulfilled. Workers at the Common Cold Unit at Salisbury have produced cold-like symptoms in volunteers with the following viruses propagated in tissue cultures or in fertile eggs: Influenza A; Parainfluenza 1 and Parainfluenza 3[1]; ECHO 11[2]; ECHO 20; ECHO 28 (JH or 2060)[3]; Coe virus[4]; Adenovirus[5].

With most of these viruses, the colds produced were not quite typical, particularly when attention was given to the whole group of inoculated persons and not only to individuals. For instance, the adenoviruses and parainfluenzas gave rise to numerous cases of fever; ECHO 11 and 20 caused gastro-intestinal as well as respiratory symptoms (usually sore throats). The Coe virus, however, produced typical colds; so too did Echo 28, though the earlier results were hard to interpret.

On the other hand, most of these viruses were not isolated from typical colds. The parainfluenzas in particular came mainly from minor respiratory infections in children, while the adenoviruses, Coe virus, and some others were most frequently from outbreaks in recruits. During fifteen years' work at Salisbury, none of these viruses has been recovered from nasal washings from typical naturally-occurring colds in adults, though such washings will reproduce colds in a high proportion of volunteers. Several strains of Echo 28 have recently been isolated, however, from washings from colds in children.

All things considered, the Salisbury workers did not feel that typical common cold viruses[6] were being dealt with. Then, in 1960, it was reported that some rather different viruses had been cultivated from colds

*Formerly, Deputy Director and Head of the Virology Department, National Institute for Medical Research, London.

in adults and that these had strong claims to be the agents which were being sought[7]. These newly discovered agents, for which the name Rhinovirus is proposed, are the subject of this paper.

PROPERTIES OF RHINOVIRUSES

(i) *Size*. Two strains have passed through gradocol membranes of average pore size 0.100μ, being retained by those of 0.69μ. This result might suggest a virus diameter of 64 mμ. However it is well known that much virus, if it is of low titre, may be retained by filters; since the virus titres in these instances were only $10^{1.5}$ and $10^{2.5}$, it is likely that the virus' true size is much less, very possibly in the range of most enteroviruses, about 25 to 30mμ.

(ii) The viruses tested have resisted treatment with 20% ether overnight and were inactivated when heated 30 minutes at 56°. They have been readily purified by blending with fluorocarbon (Arcton 23) and centrifuging.

(iii) The type of cytopathic effect in culture has been very similar to that produced by enteroviruses.

(iv) The rhinoviruses tested (three strains) have not produced symptoms on intranasal or intracerebral inoculation into suckling mice, nor has any other experimental animal proved to be susceptible to the agents.

In all these respects rhinoviruses resemble ECHO viruses and it could be argued that, when their properties have been worked out a little further, they should be allotted numbers in the enterovirus or ECHO virus series. There are, however, cogent reasons against adopting this course.

DIFFERENCES FROM ENTEROVIRUSES

The term enterovirus implies a habitat in the intestine and the "E" of ECHO virus has the same connotation. The rhinoviruses appear to multiply in the nose—hence the name; inoculation even on to the posterior pharyngeal wall is less successful than into the nose. They have not yet been isolated from faeces, though it will not be surprising if a little virus manages to survive passage through the gut now and again. Even so, the name enterovirus would seem to be wholly misleading. The rhinoviruses seem certain to form a family with numerous serological types, some already known, many other yet to be described. One can hardly imagine a course leading to more confusion than mixing them up in a numerical series with enteroviruses of entirely different habitat and pathogenicity. Fortunately, the rhinoviruses have characters other than habitat which permit their separation from ECHO or other enteroviruses. These are not fundamental, structural, stable characters such as serve to separate viruses into

their major divisions[8] but secondary characters of the kind useful in subdividing for convenience the major groups. These characters concern the conditions for growth in tissue culture and depend upon a necessity, at least on primary isolation, for (i) a lower temperature than is conventional, that is 33°C, rather than 36° or 37°; (ii) a rather acid pH in culture; and (iii) a high oxygen tension. The last is shown in part by the need to keep cultures rotating. All three conditions are fulfilled in the virus' natural habitat, the nose; and the rhinoviruses can be conceived of as enteroviruses which have become adapted to life in this environment.

So far, rhinoviruses have been cultivated only in primate epithelial cells. Most of them, the H strains, have grown as yet only in human embryonic kidney; others, M strains, also in monkey kidney and in several continuous cell lines of human origin. The M strains so far isolated fall into 2 serological types. It is uncertain how many types of H strains there will be, as it is difficult to make antisera against them; there are at least four, probably many more.

POSITION OF ECHO 28 VIRUS

Among strains recently isolated in Britain, there have been a few which behave like the M strains of rhinovirus but clearly fall, by serological tests, with ECHO 28. They differed from the other M strains in growing readily from the beginning at 36°C. Quite obviously, the ECHO 28 viruses form a link between the rhino-and ECHO viruses. There has been only one recorded isolation of ECHO 28 from faeces. The strains differ from the rhinoviruses in having less exacting temperature requirements. On the other hand they do resemble them in preferring a slightly acid medium for growth, and rolling of culture tubes. Further, washings containing these viruses have produced more typical colds than have other ECHO viruses such as 20. If the separation of rhinoviruses from ECHO viruses comes to be accepted, it might be more logical to class the JH-2060 viruses as aberrant rhinoviruses than as aberrant ECHO viruses. The point is not, however, important when we come to consider the taxonomy of enteroviruses as a whole.

CLASSIFICATION OF ENTEROVIRUSES

Only four years have passed since the general recognition that Poliomyelitis, Coxsackie, and ECHO viruses form a single family. The discussions at the Fourth International Poliomyelitis Congress held in Geneva in July 1957 made this grouping seem likely. In the summary at the end of that Congress, I said "We are beginning to see, as a whole, a family of

viruses which is characterized by a habitat in the intestinal tract of mammals, very small size, spherical shape, resistance to ether and perhaps a definite range of stability to varying pH's.'"[9]

In 1957, the American ECHO virus Committee changed its name to Enterovirus Committee, recognising that it had to deal with one virus family[10]. Since about that time, it has been growing harder to draw the line

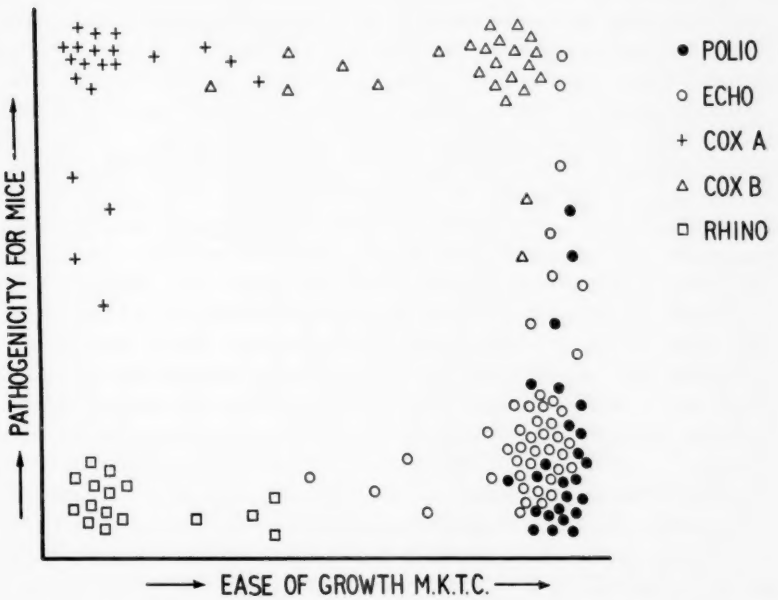

FIG. 1. The viro-astronomical approach to classification: grouping of certain "*Nanivirus*" constellations based on their pathogenicity for suckling mice and ease of growth in monkey kidney tissue cultures.

between Coxsackie and ECHO viruses. The main distinction lies in pathogenicity or otherwise for suckling mice; yet there are representatives of single serotypes, some of which will kill baby mice and others will not; or again some will do this when freshly isolated, others after varying numbers of mouse-passages, others not at all. The distinction is so illogical that many would like to abandon the attempt at separation and to call them indiscriminately "enteroviruses". It may become equally hard to distinguish between polioviruses and other enteroviruses, a distinction based chiefly on pathogenicity for the central nervous system of primates. This, too, is illogical, for attenuated poliovirus strains no longer possess this distinguishing

character. And there is a virus which Russian workers[11] have called poliovirus Type IV but which is recognised elsewhere as Coxsackie A type 7.

There is an alternative to abandoning the well-known groupings into poliovirus, Coxsackie A, Coxsackie B, and so on. I call this the viro-astronomical approach. Astronomers perceiving a sky dotted higgledy-piggledy with stars did not abandon the effort to classify them but grouped them, quite illogically, into constellations, groupings which are still useful after hundreds of years. Similiarly, enteroviruses can be grouped into constellations by considering together any two properties, say the ability to grow in monkey kidney tissue culture and pathogenicity for suckling mice. If this is done as shown in figure 1, we see in the right hand lower part of the chart a cluster of viruses readily cultivated in monkey kidney, but of low pathogenicity for baby mice; these are the polio-and ECHO viruses. At the upper right are the Coxsackie B viruses, most of which can be cultivated in monkey kidney and which are also pathogenic for mice; at the upper left are Coxsackie A viruses, growing well in mouse brains, but poorly in monkey kidney cultures. Finally the rhinoviruses (lower left) do well in neither respect. The figure shows, however, that the differences are not absolute: some polioviruses will infect mice; some Coxsackie A viruses will grow in monkey kidney cultures, as will some rhinoviruses. A similar chart could employ any other two useful characters; for instance use of monkey pathogenicity would serve to separate polioviruses and ECHO viruses. However, it is not likely that use of more than very few characters would be necessary to separate all the important groups. We may conclude that it is probably fruitless to try to put every "new" enterovirus definitely into one group or another. Nevertheless the names currently used to denote clusters or constellations of viruses still have a significance and a worthy place in virology. After all we find the terms hillock, hill, and mountain very useful, even though we cannot sharply distinguish between them.

THE NANIVIRUSES

It is worth looking a little more widely than just at the enteroviruses affecting man. These seem to be members of a large natural family of viruses, all small, ether-resistant viruses which so far as is known contain RNA and have a similar structure. Besides the viruses already discussed there are those causing Teschen disease of pigs, encephalomyelitis of mice, epidemic tremor of chickens, and the very many viruses isolated from the intestinal tracts of monkeys, pigs, cattle, cats, and birds. Those unassociated with disease have been named according to the host species ECMO (enteric cytopathic monkey orphan), ECSO (in swine) ECBO (in bovines) etc.

on the analogy of ECHO. Such names can have but ephemeral value for there are but 26 letters in the alphabet and many thousands of known vertebrates. There is also the virus of foot-and-mouth disease, which, incidentally, in its pathogenic effects on mice, closely resembles Coxsackie virus[12]. Finally there are the encephalomyocarditis (EMC) viruses differing from the others in their pathogenicity for a wide range of hosts.

The characteristic feature of all these agents is their small size; hence the suggested name *"Nanivirus"* from the Latin *nanus* = a dwarf[8]. If one looks at these as a whole, it becomes much more reasonable to place within the large family a section—rhinoviruses—having the characters described earlier. It will then appear that it does not matter greatly if they grade into the ECHO viruses, for these in turn grade not only into Coxsackies but also into ECMO, ECSO, etc; ECSO into Teschen virus and so on.

With an agglomeration of viruses such as this, the taxonomist who would designate and name species is confronted with an almost impossible task. The criteria he has for subdividing his major groups are largely characters, such as specificity for particular hosts or culture-systems, which are rather readily manipulated in the laboratory. In the writer's view the right course to pursue at present regarding nomenclature is to give names to only a few major, fundamentally-different, groups of viruses. These could be validated by the international nomenclature committee. Names for lesser groupings, such as rhinovirus and enterovirus, will prove useful for some time but should not be officially blessed for the present.

There is a practical aspect of all this. With all the many new viruses that are being discovered, there is bound to be confusion if workers in different countries give different names to the same virus. The World Health Organization has accordingly begun to designate International Reference Laboratories in hope of keeping these matters under control. A beginning has been made with an Enterovirus Reference Laboratory at Baylor University in Texas and a Respiratory Virus Reference Laboratory at Salisbury in England. If enteroviruses are claimed to cause respiratory infections, there could arise difficulty in determining the spheres of influence of different laboratories within the virus field. Fortunately it is more likely that there will be fruitful co-operation in this matter between workers in many lands. Such co-operation will be helped if the viruses concerned can be viewed in some sort of taxonomic proportion.

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F. M. BURNET*

The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia

CELLULAR ASPECTS OF IMMUNOLOGY AS MANIFESTED IN THE SIMONSEN REACTION

Immunology has traditionally been concerned with two central topics—immunity against pathogenic microorganisms and the nature and properties of antibodies. Both of course are, and will remain, immensely important but at the present time there can be little question that the problems of the origin, nature, and behaviour of the "immunologically competent cell" provide the centre of current interest in theoretical immunology. There are five sets of experimental findings that are chiefly responsible for that interest and which lay down the lines on which modern theories of immunity have been developed.

They are:

i) The fact that in a child with congenital agammaglobulinemia who cannot produce any type of conventional serum antibody, measles infection runs a normal course and is followed by specific immunity against reinfection[1].

ii) Algire's evidence that homograft immunity is mediated by cells not antibody[2].

iii) Evidence by Coons *et al*[3]., White[4], and others that the cells responsible for antibody production are plasmacytes (immature or mature) which are present in clone-like accumulations. In at least the great majority of cases they produce antibody against one antigen only.

iv) The fact that a wide range of immunological capacities can be transferred by cells but not by serum from an actively immunized animal to a normal recipient. These include the capacities to show delayed hypersensitivity, to produce antibody on secondary challenge, and to protect animals whose resistance has been destroyed by irradiation.

v) The phenomena of autoimmune disease point strongly toward the interpretation that tissue damage is due to pathogenic cells not to the direct or indirect action of antibody.

Immunology has perforce been largely concerned with antibodies because of the ease of recognising, handling, and titrating antibody. The evidence

* Director, The Walter and Eliza Hall Institute of Medical Research.

for the existence of immunologically competent cells is, in fact, nearly always obtained indirectly from antibody measurements of one sort or another. Particularly for the further study of the autoimmune disease, but also because of its bearing on the general theory of immunology, one of the major present-day needs is to find ways of recognising immunologically competent cells according to their specific reactivity—in a fashion analogous to the way that a solution of gamma globulin can be identified as, e.g. an immune agglutinin. To date the only way of doing so has been to use Coons' fluorescent antibody "sandwich" technique, or some analogous method using a highly recognisable antigen. By these methods it has been possible to show what type of antibody a given plasmacyte was making, the reactions being essentially those of a rather large concentration of antibody molecules held in some fashion on the cell surface.

The chief interest of the experimental work I wish to describe is that it may represent a step toward the functional analysis of a population of mesenchymal cells in terms of specific immunological capacities. For the last few years work on the Simonsen phenomenon, i.e. the production of lesions in chick embryos after inoculation of mature blood or spleen cells from the same species, has been studied by a series of workers in this laboratory*. The present account makes use of contributions by all of these workers but the interpretation offered is a personal one.

The essentials of the phenomenon can be presented by considering a single experiment which has already been reported and illustrated[5]. Using a closely inbred strain of fowl, four embryos in eggs laid by one hen were inoculated on the chorioallantoic membrane (CAM) with blood leucocytes from the cock used for artificial insemination. The four membranes showed respectively 203, 110, 0, 0, opaque white foci when they were harvested four days after inoculation. Related experiments with the same stock of birds indicated that the cock in question could be designated genetically CC and the hen as BC. Both CC and BC offspring would be expected and the result is interpreted as representing 1:1 segregation. CC leucocytes on CC membrane produce no lesions, on BC membranes containing an antigen not represented in the donor 100-200 lesions were produced.

The focal lesions average about 0.5-1 mm. in diameter and are easily counted with the naked eye. Their histological structure has been described[6]. Essentially they are composed of embryonic host cells, most of which in the early stages result from local proliferation but later include many cells from the blood. In addition there are less numerous cells with some

* The workers include Peter Isacson, Georgiana Boyer, F. M. Burnet, Patricia Lind, Alexander Szenberg, Noel Warner, and Deborah Burnet.

resemblance to plasma cells which we have provisionally identified as descendants of the initiating donor cells. The pathogenesis of the lesion is interpreted as follows: An immunologically competent cell from the adult chicken blood reacts with the foreign antigenic determinant present in the embryonic tissues to release pharmacologically active material which can stimulate adjacent host cells, epithelial and vascular endothelial, to proliferate irregularly. It is not established whether the same agents stimulate the donor cells to proliferate or whether these have an intrinsic ability to do so in the embryonic environment. In any case with proliferation of the immunologically competent cells new contacts with the foreign antigenic determinant stimulate further proliferative activity and eventually the lesion becomes a dense mass of pyknotic and dehydrated cells. Movement of descendant donor cells can give rise to new lesions, either local satellites or distant "metastases", particularly in the embryo's spleen.

In this experimental system we have an indication of the immunological potentiality of single cells and our central interest has been to attempt an analysis of the qualities of the cells responsible for the initiation of specific foci. It is not possible to isolate a single cell and show that it is responsible for the initiation of a lesion but all our experience plus the analogies of many basically similar types of biological quantitation, is in line with the interpretation that *one* cell can induce a focus. Since only a small proportion of cells actually initiate foci, information about their essential qualities must be gained indirectly and in the next section a summary is given of the relevant experimental findings.

PROPERTIES OF COMPETENT CELLS

1. *Histological type of cells in blood.* Szenberg and Warner[7] have made extensive studies of the relation between the number and type of leucocytes in the blood and the focal counts obtained on membranes of randomly bred chick embryos. In agreement with earlier estimates they found about one focus per 20,000 leucocytes but when differential studies were made a relatively close correlation emerged between the count of large (immature) lymphocytes and the median number of foci. The ratio was around 2500 large lymphocytes per focus and it is noteworthy that on rare occasions individual membranes appeared to indicate that as few as 100 large lymphocytes—in one instance 40—could produce one focus. These results make it reasonably certain that a competent cell in the form of a large lymphocyte is more likely to produce a focus than if it is a small lymphocyte or a granulocyte and it may well emerge that the large lymphocyte is the only effective cell. It is equally evident that there are substantial differences

in the "plating efficiency" of the process from one embryo to another. In random embryos this may be due in part to genetic and antigenic variability but even in the example quoted earlier, two embryos of identical genetic quality gave a twofold difference in the counts of foci. A point on which considerable emphasis should be laid is that if the large lymphocyte is the only type of cell which is effective, the minimum number of cells which can produce a focus is considerably smaller than would be expected on any unsophisticated clonal theory of immunity.

TABLE 1. DEVELOPMENT OF COMPETENCE TO PRODUCE FOCI WITH AGE

Age	*No. tested*	*Leucocytes x 10^6/ml.*	*Negative membranes*	*Median counts of foci from individual bloods*	*Foci from spleen suspensions*
19 d. embryo	3	1.5-2.6	22/22	—	0
Hatched chickens					
12 hours ±	4	0.6-2.0	22/22	—	0
24-30 hours	7	0.2-1.0	27/53	0.3,* 0.3, 1.0, 1.1, 1.3, 1.4, 1.5	+
2 days	2	0.4-0.6	5/16	0.6 7.5	+
3 days	3	1.4-2.3	3/29	8, 10, 10	
4 days	3	1.3-5.2	2/30	2.5, 5, 10	
6 days	2	2.0-2.2	1/22	5, 6	

* Counts below 2 are from series with a proportion of negative membranes and are means.

2. *Activity of cells from lymphoid organs.* Spleen cell suspensions from grown chickens produce foci similar to those obtained from blood leucocytes. If the large lymphocytes are accepted as the significant cells, they have approximately one-fifth the activity of similar cells in the circulating blood[7]. One focus is produced per 10,000-15,000 large lymphocytes from the spleen. The few foci produced with suspensions of lymphoid cells from thymus or bursa of Fabricius could be ascribed to a small contamination with blood cells. If the large lymphocytes from these organs are effective at all it must be at a very low level.

3. *Maturity of cells.* Embryonic blood or spleen cell suspensions give no foci on the CAM of unrelated embryos but the capacity develops very soon after hatching. Results obtained by D. Burnet on this point are shown in Table 1. Virtually full competence is obtained by the third day after hatching. Since in all experiments normal untreated birds are used as donors of competent cells, there is an *a priori* assumption that no previous immuno-

logical experience of the donor is relevant. The fact that cells from chickens less than a week old are effective reinforce the validity of this assumption.

4. *Rapidity of initiation of the lesion.* In a responsive membrane lesions can be seen macroscopically at 44 hours after inoculation and histological examination of two, three, and four day lesions makes it necessary to assume that the process is initiated very soon after the effective cell has lodged on the CAM. If each cell generation requires eight to twelve hours there is only just enough time to account for the observed accumulation.

5. *Immunogenetic aspects.* Evidence has been published that when blood from a grown bird genetically identical with the embryo is placed on the

TABLE 2. ACQUIRED TOLERANCE OF AN AA CHICKEN INJECTED "IN OVO" WITH EMBRYONIC CC CELLS AS SHOWN BY TESTING BLOOD TAKEN AT 12 DAYS AFTER HATCHING ON CAM'S OF KNOWN GENOTYPE

Genotype of test embryos	*Focal counts*
AA	0, 0, 0
CC	1, 1, 0, 0
AB	16
Random	++, 13

++ Lumpy uncountable lesion.

membrane no foci are produced[8]. Experiments in progress (D. Burnet) have shown that when 16 day embryos of AA genotype received, intravenously, a pool of cells from CC embryos of similar age, tolerance is produced. Blood from such a chicken taken 12 days after hatching gave the results shown in Table 2.

Limited experiments with randomly bred and pure line chickens inoculated with chick embryo cells have given no evidence to suggest that the number of competent cells in the blood can be increased by such attempts at immunization.

In comparing results obtained with pure line cells on randomly bred embryos as against the counts on embryos differing from the donor by a single genetic (antigenic) factor, no significant difference was evident in the median counts.

6. *Action of Colchicine.* Our hypothesis of the action of the cells concerned in initiating the CAM lesions is that very shortly after lodging on the membrane they begin to proliferate. It might be expected therefore that if the completion of mitosis were prevented by treatment with colchicine, the

number of foci would be diminished. In unpublished experiments by D. Burnet[9], heparinized chicken blood was treated with graded doses of colchicine in lightly buffered saline containing antibiotics for one to two hours at 37°. The cells were then washed in saline and inoculated in a series of embryos. The results are shown in Table 3. Using a dose of 0.01 mg./ml., no significant difference was found between the effect at one hour and two hours. There is a significant reduction in focal count therefore on exposure of the cells to quite small concentrations of colchicine.

7. Passage of competent cells. It has been shown that by appropriate treatment, cells capable of producing foci on a new CAM can be extracted

TABLE 3. ACTION OF COLCHICINE "IN VITRO" ON COMPETENCE OF CHICKEN LEUCOCYTES

Concentration of cholchicine (mg./ml.)	*Time of exposure*	*Median focal count*
0.01	1 hour	5.1^{21}
0.005	1.5	9.2^{22}
0.001	1.5	10.3^{21}
0.0001	2	14.2^{17}
Control		28.0^{18}

Superscripts show number of membranes counted.

from the four-day CAM lesions[10] but it is technically much more satisfactory to carry out passage experiments using cells from embryo spleens. Following inoculation of adult blood and lesion production on the CAM, the spleen will usually show lesions by seven days but most of our passage experiments were initiated by intravenous injection of blood leucocytes and continued by the injection of spleen cell suspensions by the same route. At each stage focal counts on the CAM were obtained for the inoculum used intravenously. With five day intervals, first passage of the primarily infected spleen gave large numbers of foci with a characteristic variability in size; with subsequent passage, the number and size of foci diminished and no foci were obtained after the fourth. Experiments of this type have so far been confined to randomly bred embryos.

When pure line donors are used it is possible to show that changes in the qualitative competence of the cells take place with a single passage. With the stock available in 1959, about 50 per cent of A type leucocytes, tested on embryos of the corresponding line, gave zero counts. If such leucocytes were injected intravenously into a randomly bred embryo and a cell suspension from the resulting splenic lesion tested on pure line embryos,

100 per cent of the CAM's showed lesions[11]. If the competent cells in the spleen were actually descendants of the donor cells, they had lost their inability to produce lesions on genetically identical embryos. From both points of view therefore it is clear that passage results in changed characters for at least a proportion of the descendants of the initiating donor cells.

THE INTERPRETATION OF THE SIMONSEN PHENOMENON

The Simonsen phenomenon is a typical example of a graft-versus-host reaction and its interpretation must follow the pattern for similar mammalian phenomena. The fact that single cells are by implication concerned may make it simpler to see the general significance of the graft-versus-host reactions for immunological theory.

In the discussion we are concerned only with the process by which immunological activity, in this case lesion formation, is initiated by a single cell. The possibility that we are not concerned with activation and proliferation of a single cell as the initiating process will be regarded as too remote for serious consideration. From the point of view of experimental verification, the comparison must necessarily be between some form of clonal selection theory on the one hand, and those theories which confer a potentiality to develop *any* pattern of immunological activity on any physiologically suitable cell, on the other.

The essence of a clonal selection theory is that a population of immunologically significant cells from a normal animal can be divided into subpopulations which differ in their specific immunological reactivity. A formulation in terms of clonal selection could take several different forms:

i) the original form in which each cell or clone carried one or at most two random patterns. A very large number of individual clones must be postulated,

ii) a larger number of patterns, still arising at random on each clone. This requires more *ad hoc* assumptions but can reduce greatly the number of clones required.[6]

iii) a more or less determinate distribution of specific patterns as part of the process of differentiation.

The essential feature of all these variants of the theory is that cells or clones from normal animals have only a limited number of preadapted potential reactivities in terms of specific immunological pattern.

The instructive or subcellular selective theories all assume that a potentially competent cell may, by appropriate contact with antigen, take on any one of a virtually unlimited number of immunological patterns and that all

such cells are immunologically equivalent. In the normal animal such cells can be regarded as having no specificity until they have had contact with an antigen.

Superficially considered, the production of foci on the CAM is most simply interpreted on the basis of a clonal selection theory of immunity.[19] In previous discussions[18] it has been suggested that each grown chicken has, in its blood, lymphocytes preadapted to react with all potential antigens which are *not* present in its own cells and fluids. On contact with a CAM in which a "foreign" antigen is present, any cell preadapted to react with that antigen will, if other conditions are propitious, be stimulated to proliferate and to induce associated proliferation of host cells.

Points raised in favour of this were (i) the fact that *normal* cells were concerned, (ii) the early initiation of the lesion indicates that there is a rapid "recognition" of the presence or absence of a foreign antigenic determinant, (iii) the small proportion of cells actually initiating a lesion.

On the other hand, there are several observations which are not easily fitted into such a picture and point rather to a lack of specific preadaptation in the initiating cells. These include the following findings:

1) The number of large lymphocytes per focus can be considerably lower than is consistent with a random distribution of all possible immune reactivities.

2) There is no larger number of foci produced on membranes differing presumably by many antigenic components from the leucocyte-donor than on membranes differing only by one antigen.

3) Passage of competent cells through an embryo results in a change in their postulated specific reactivity.

The problem presented then is to determine which of the two main alternatives is the most satisfactory to cover the facts and if one can be chosen what modifications are necessary. Modern immunological discussion tends to be based on two axioms: i) Crick's dogma that genetic information is expressed in specific protein structure by a process moving from DNA to RNA to protein—not in any other sequence, and ii) a normal animal can develop no immunological reactivity against components present in the "accessible" regions of its own structure. Not-self is differentiated from self by its ability to provoke an immunological response.

The first axiom demands some form of elective theory but does not exclude the limiting form of the subcellular selection hypothesis by which a cell can be stimulated by any antigen to make the corresponding antibody or develop specific reactivity against it.

We are driven therefore to start the discussion from the angle of self-recognition. The immunogenetic results are perfectly clear. Cells from a bird of genotype AA will produce no foci on CAM's of AA embryos, but a full quota of foci on AC or CC membranes. By pretreatment in the embryonic stage, an AA chicken can be rendered tolerant to CC cells and its leucocytes can now produce no foci on either AA or CC membranes but the normal number on an AB membrane.

It is clear that the population of cells put on the membrane in one way or another carries the information allowing it to distinguish whether the CAM contains only antigens with which the organism has been in contact during embryonic life or whether a foreign antigenic determinant is present.

The demonstration of acquired tolerance eliminates the alternative statement that the cell can recognise genetic similarity unrelated to antigenic structure.

On clonal selection theory any cells which could react with self antigens have been eliminated. Among the remainder there are cells preadapted to react with any histocompatibility antigen not present in the donor. It is from such cells that the foci are initiated.

If we reject clonal selection, the alternative possibilities are:

1) The reactive cells are there in virtue of their having acquired the capacity to react with foreign (but related) histocompatibility antigens by some determinate process, not by a purely random distribution of all possible patterns.

If we adopt any form of subcellular selection theory we must assume that any receptors on cells which could react with self-components are either absent or blocked at the relevant time while a receptor capable of reacting with the foreign antigenic determinant is present and functional. These requirements could conceivably be fulfilled in such a way as to allow any functionally active lymphoid cell to react with a large variety of foreign antigenic determinants.

3)A third possibility has been raised in discussion, viz. whether the recognition process may not be something of a non-immunological character. If the donor cell is genetically distinct it may encounter a cell surface which has an irritant quality that would be absent if donor cell and CAM cell were genetically equivalent. Multiplication of the cell takes place and at some stage specific immune activity is developed against the foreign antigen which is fortuitously present. In some way the cell can recognise in a positive fashion that it is on "self-territory"; anything other than self-territory has a nonspecific irritant action. The

implication is that there is a recognition in the sense that some pattern carried by the donor cell meets and reacts with another complementary pattern carried only by cells of precisely similar genotype to that of the donor. This is basically similar to the self-marker hypothesis of Burnet and Fenner[14] and gives rise to the same objections as led to our disuse of the concept. It seems most likely that any self-marker capable of changing with every minor antigenic change could be anything other than the histocompatibility pattern itself.

Another possibility which differs only slightly from the first is that all physiologically appropriate cells start proliferating as soon as they implant on the CAM but only those which can develop an immunological response to any antigens which can be recognised as foreign will initiate a focus. Here the only gain is to provide a slightly more generous time scale for the initiating process. The need for recognition of the antigen as foreign persists.

The existence of acquired immune tolerance rendering the donor's cells nonreactive on what would genetically be an appropriate CAM for lesion formation cannot be interpreted except along immunological lines. Surely, other genetic qualities of the cells cannot have altered. In addition the evidence is adequate that the difference between donor cell and host is qualitatively the same as the difference responsible for homograft reactions to skin transplants.

We can only conclude that the phenomenon of recognition shown in the experiments with inbred chickens is immunologically based and demands that in the cells responsible for the initiation of foci there are preadapted patterns complementary to the histocompatibility antigens by which the host CAM differs from the donor. A selection theory is required in one form or another—the question is what form it should take, clonal or subcellular?

In all discussions of clonal selection theory, it has been emphasized that the special virtue of the simple form of the theory was that it could be experimentally *disproved*. While it might be possible to devise *ad hoc* reasons by which the difficulties encountered in fitting the Simonsen reaction into the pattern might be overcome, the overall picture indicates that a straightforward clonal selection theory does not give an acceptable interpretation of the phenomena.

To account for the relatively small number of large lymphocytes per specific focus would demand a non-random distribution of patterns amongst the cell population. The fact that there is no clear increase in the number of foci on embryos differing by several antigenic factors over the number on embryos differing from the donor by only one factor, requires some rather

special pleading for its explanation on the assumption that a subpopulation of competent cells is available for each antigenic determinant. Finally the change in specificity of competent cells in the course of proliferation on passage indicates at least that any immunological pattern initially carried is highly labile. The first of these three difficulties is probably the most important one. It necessitates the assumption of an undue frequency of receptor patterns corresponding to the histocompatibility antigens of closely related stocks of the same species. It is possible that future study of the processes of morphological homeostasis[5] may give a reason why this should be so but at present there is no such justification.

Even if we can still contend that no "instructive" theory of immunity has been able to interpret the non-antigenicity of self components, we are nevertheless left with the conclusion that any process of cellular selection must be much more labile and complex than that put forward in 1959 if it is to account for the specific qualities of the Simonsen phenomenon on the CAM of the chick embryo.

Clearly it will not be possible to provide a new formulation until more data are available The most desirable approach would be to find a way by which large lymphocytes could be cloned *in vitro* by Puck's technique without loss of their immunological potentialities. Present indications are that this will prove impossible but the difficulties may well turn out to be merely technical ones. It would be premature to state alternative ways of accounting for the apparent excess of effective cells in Szenberg and Warner's experiments, until the possibility of a *determinate* distribution of patterns in the lymphoid cells can be excluded. If we are driven to the conclusion that individual cells must have a large number of different potential patterns of immunological reactivity we must still hold that "recognition" is logically inconceivable without a means of comparing a pattern from outside with another pattern held in an internal store. There are two ways by which the store of information carried by a cell could attain the necessary amount.

i) by a rapid sequence of changing patterns only a small number of which are present at any given moment,

ii) by the accumulation of large numbers of receptors or recognition units in the cell.

Whether either of these hypotheses opens up an opportunity for experimental test is a problem for the future.

CONCLUSION

The Simonsen reaction provides us with a model system with the almost unique advantage of allowing an assessment of the immunological reactivity

of single lymphoid cells. The investigations carried out in our laboratory which I have reviewed in the present paper indicate that,

1) Individual cells are capable of "recognising" the presence of a foreign antigen.

2) Quantitative results are inconsistent with this recognition being the prerequisite only of a small proportion of cells preadapted, by some random process, to react with any foreign antigenic determinants present in the embryonic host.

The problem of the recognition of "being foreign" by the normal lymphoid cells of the vertebrate organism still remains the major difficulty of immunological theory. The clonal selection theory was devised primarily in an attempt to resolve the difficulty but the results of the Simonsen reaction studies show that if the theory is to account for primary immune responses it must be reformulated in some more sophisticated form.

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EDWARD C. CURNEN*
HARRIE R. CHAMBERLIN**

Department of Pediatrics, College of Physicians and Surgeons, Columbia University and the Pediatric Service, Babies Hospital, Columbia-Presbyterian Medical Center, New York.
Department of Pediatrics, School of Medicine, University of North Carolina and the Pediatric Service, North Carolina Memorial Hospital, Chapel Hill

ACUTE CEREBELLAR ATAXIA ASSOCIATED WITH POLIOVIRUS INFECTION†

The term acute cerebellar ataxia is used to designate a syndrome of uncertain etiology, occuring usually in young children and characterized by a relatively sudden onset of cerebellar signs (ataxia, intention tremor, and often nystagmus) which reach maximum intensity within a few days and then subside.[1-7] In many of the reported cases an absence of prodromal symptoms, fever, nuchal rigidity, or pleocytosis in the spinal fluid has been noted. Although a similar clinical picture may be encountered following exanthemata, especially varicella, or overdosage of certain drugs such as diphenylhydantoin, and as a feature of disease accompanied by more extensive neurologic signs, the designation is usually applied only when these conditions are not in evidence.

Despite considerable speculation that a virus might produce this syndrome only rarely has there been laboratory evidence of a specific viral infection in association with signs which are exclusively or even predominantly cerebellar.[1, 8-10] Poliomyelitis virus has been mentioned as a possible etiologic agent,[8, 11-13] but confirmatory evidence has been meager and isolation of poliovirus has been reported infrequently from patients in whom cerebellar signs predominated.[1,8,14] Among these two[1] appeared to fit the strictest requirements for a diagnosis of acute cerebellar ataxia, namely a sudden onset of symmetrical cerebellar signs without prodromata, fever, nuchal rigidity, or abnormalities of cerebrospinal fluid.

In this paper four additional cases of acute cerebellar ataxia associated with poliovirus infection are presented. Of these, the first two appear to

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* Professor of Pediatrics. Formerly Assistant Professor of Preventive Medicine, Yale University School of Medicine, 1946-1948; Associate Professor of Pediatrics and Preventive Medicine, Yale University School of Medicine, 1948-1952.

** Associate Professor of Pediatrics.

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satisfy strict criteria for diagnosis of the disorder. The other two conform to a less rigid but frequently acceptable definition. However, they also had other manifestations which aroused suspicion of poliomyelitis on clinical grounds alone. The significance of acute cerebellar ataxia as an entity and the association of poliovirus infection with this syndrome will be discussed.

CASE REPORTS

Case 1. B.L., a 6-year-old white boy, was admitted to the Grace-New Haven Hospital on 31 August 1949 because of "severe dizziness" of approximately 14 hours duration. Previously well, he had awakened with this complaint during the night. He had gradually developed generalized shaking of the whole body and found that he was unable to walk.

Examination revealed an alert, well-oriented, apprehensive boy who clutched his bed tightly and was unable to sit without support. His temperature was 37.8° C. A coarse, irregular tremor of the head, trunk, and all limbs was stimulated when voluntary motion was attempted, and all movements were markedly ataxic. Gross and rapid jerking movements of the eyes were conspicuously present. These movements were variable, conjugate and mainly, but not exclusively, in the horizontal plane; they were increased by movement of the head or trunk and were completely irregular in frequency and amplitude with no fast or slow component. The optic fundi appeared normal. Moderate dysdiadochokinesis was noted. Finger-to-nose testing revealed considerable increase in the tremor on intention. The superficial reflexes were present, the deep tendon reflexes were slightly hyperactive and symmetrical, and the plantar responses were flexor. No evidence of nuchal rigidity or muscular weakness was detected.

The results of urinalyses and routine hematologic studies were not remarkable. The erythrocyte sedimentation rate on the fourth hospital day was 9 mm./hr. Examination of cerebrospinal fluid on three occasions revealed 0-3 lymphocytes/cmm. and protein levels of 10-15 mg.%. The sugar content was normal and cultures showed no growth. X-ray films of the skull and an electroencephalogram disclosed no abnormalities.

The patient's course was one of steady improvement. Initially he vomited occasionally. His temperature was never significantly elevated. He experienced no headache, stiffness of the neck, dysphagia, dysarthria, earache or tinnitus. Subjective dizziness was gone by the third day although when discharged from the hospital on the eighth day he still had gross ataxia of the trunk and walked with an unsteady, broad-based gait. On the fourteenth day he exhibited only slight unsteadiness on toeing a line. When he was seen again on the twenty-eighth day, all abnormal signs had disappeared.

Case 2. L.R., a 20-month-old Negro girl, was admitted to the North Carolina Memorial Hospital on 11 April 1960, because of inability to walk. Seven days earlier, marked generalized shaking had first been noted when she tried to get out of bed. This condition had persisted, except when she lay completely still, and had possibly increased for she could no longer walk. Jerking movements of her eyes were also present. She had coughed and had some rhinorrhea intermittently for several weeks earlier but otherwise had been well and had not had fever. She had received no injections for immunization against poliomyelitis.

Examination revealed an unhappy, apprehensive child with a severe, coarse, ataxic tremor on movement of her trunk, head, or limbs. Her temperature was 37.8° C. by rectum. She was unable to sit without support. Any attempted motion of an arm greatly increased the tremor. Constantly varying, conjugate, coarse, jerky eye movements with no fast or slow component were present on gaze in any direction and increased by superimposed volitional movements of the eyes. Abdominal reflexes were present, the deep tendon reflexes were symmetrical and physiological, and the plantar responses were flexor. Nuchal rigidity and muscular weakness were not demonstrated.

Hematologic studies revealed a moderate microcytic, hypochromic anemia (hemoglobin 8.0 gm. %) but otherwise normal findings. A sickle cell preparation was negative. Urinalysis was normal. No ova or parasites were detected in the stools. A culture of the nasopharynx yielded hemophilus influenzae. Examination of cerebrospinal fluid revealed 3 lymphocytes/cmm., 22 mg.% of protein and 40-50 mg.% of sugar; cultures showed no growth. X-rays of the skull were not remarkable and an electroencephalogram was normal. An intracutaneous tuberculin test with P.P.D. was negative.

The patient remained afebrile and was discharged after four days, on sulfadiazine and ferrous sulfate, with no definite improvement in her neurologic signs. Three weeks later the abnormal ocular movements had disappeared and the ataxia had improved, so that she could crawl and take a few steps when supported. She finally was able to walk without assistance seven months later. When seen fifteen months after the illness her gait was still slightly ataxic, and a moderate, coarse intention tremor of the arms had persisted although she could now manage a glass without spilling.

Case 3. R. T., a 6-year-old Negro girl, was admitted to the North Carolina Memorial Hospital on 24 October 1959, because of a staggering gait. Three days earlier she had complained of headache; on the next day she also experienced abdominal discomfort and possibly had slight fever. At this time a staggering gait was first noted, which had persisted, without apparent weakness. She had not received any injections of poliovirus vaccine.

On admission her temperature was 38.3° C. by rectum. She was irritable, and walked with a rather rapid, broad-based, unsteady gait, tending to fall to the right. A mild tremor of her outstretched hands was noted. No nuchal rigidity, no nystagmus, no increase of the tremor on intention, and no apparent weakness were detected. Abdominal reflexes were present. The deep tendon reflexes were somewhat brisk but symmetrical and the plantar responses were flexor.

Initially the total leukocyte count was 14,300 with 69% polymorphonuclear leucocytes; this returned to normal two days later. The hemoglobin was 13 grams; urinalyses were normal and a sickle cell preparation was negative. Cultures of the nose and throat yielded no pathogens. X-rays of the skull were not remarkable. A lumbar puncture, probably traumatic, yielded cerebrospinal fluid with 1180 fresh red cells/cmm. and 75 white cells/cmm., 86% of them mononuclear. The protein content was 22 mg.%. An intradermal tuberculin test with P.P.D. was negative.

On the morning following admission the patient's neurological abnormalities had disappeared. She was able to walk and run with ease; the tremor was gone. Her rectal temperature rose to 38.1° C. that afternoon but was normal thereafter. She was discharged on the fourth hospital day. On return, one month later, she appeared entirely well with a normal gait and symmetrical, normally active deep tendon

reflexes. A physiotherapist, however, detected slight weakness about the right hip and knee. The patient failed to keep further appointments, but her parents claimed that a year after her illness there was no weakness to be noted and that she seemed completely well.

Case 4. M.S., a 22-year-old white female college student, was admitted to the North Carolina Memorial Hospital on 25 September 1956, because of fever, headache, dizziness, and nystagmus. Four days earlier she had developed fever, malaise, anorexia, and dizziness. After two days, these complaints and the onset of severe headache brought her to the university infirmary where nystagmus was noted and a lumbar puncture was performed. The cerebrospinal fluid contained 5 lymphocytes and 8 polymorphonuclear leukocytes/cmm. A Pandy test for protein was positive.

Four years earlier she had experienced a febrile illness associated with temporary left hamstring weakness, diagnosed as poliomyelitis. She had never received poliomyelitis vaccine.

Examination revealed a mildly apprehensive young woman with a rectal temperature of 38.1° C. Nuchal rigidity had not been observed prior to the initial lumbar puncture, but was present on admission to the hospital. The nystagmus was horizontal and slightly rotary on looking to the right, horizontal on looking to the left, and horizontal to the right on looking upward. A fine titubation of the head was present and an intention tremor of fast frequency and low amplitude, noted in the upper extremities was more pronounced on the left. No muscular weakness was detected and deep tendon reflexes were normally active and symmetrical.

The results of routine examinations of blood and urine were not remarkable. A second lumbar puncture was performed on the fourth hospital day; the cerebrospinal fluid contained 36 leukocytes/cmm., 80% of them lymphocytes. A quantitative determination of protein was not done. The Pandy reaction was negative. Serologic studies carried out later revealed no heterophile antibody; the results of complement fixation tests for eastern and western equine encephalomyelitis, St. Louis encephalitis, lymphocytic choriomeningitis, and mumps viruses were all reported as negative.

During the first three hospital days the titubation of the head and the intention tremor increased considerably, the latter spreading to involve all four extremities but continuing to be most marked in the left arm. The nystagmus persisted. During the early period of acute illness the patient's gait was not tested. On the fourth day minimal right facial weakness, slight inequality of palatal movements, and mild deviation of the tongue to the left were noted; all but the last disappeared within a few hours. She was afebrile on the fifth hospital day and all symptoms and signs gradually improved during the twelve-day period of hospitalization. A detailed muscle evaluation prior to discharge revealed no weakness other than the slight deviation of the tongue.

On re-evaluation a month later the patient gave an interim history of easy fatigability, excessive irritability but a decrease of the difficulty with her handwriting experienced earlier. On several occasions while reclining she had felt a sudden generalized contraction of her body. On examination she showed slight residual nystagmus on looking to the left, minimal weakness of the left side of her tongue, minimal titubation of the head, and continued slight intention tremor, more pronounced in the left arm. Subsequently all signs and symptoms gradually subsided. Fourteen months after the onset of her illness she reported that for the preceding 6 months she had been entirely well.

TABLE 1. CLINICAL SIGNS IN PATIENTS WITH ACUTE CEREBELLAR ATAXIA

Patient					Signs								
					Acute						Residual		Duration (months)
Case no.	Age yrs.	Race	Sex	Date of onset of illness	Ataxia	Tremor of head and neck	Tremor of extremities	Abnormal ocular movements	Nuchal rigidity	Muscular weakness	Cerebellar	Muscular weakness	
1	6	W	M	8/30/49	+	+	+	+	0	0	0	0	<1
2	1⅔	N	F	4/4/60	+	+	+	+	0	0	+	0	>15
3	6	N	F	10/21/59	+	0	+	0	0	±	0	±	<1
4	22	W	F	9/21/56	±	+	+	+	+	+	0	0	<8

CLINICAL AND EPIDEMIOLOGIC OBSERVATIONS

Noteworthy clinical signs observed in the four subjects of this report are summarized in Table 1. Further reference to these signs and other features of disease in these cases will be made in the discussion to follow.

The acute illness experienced by these four patients appeared to be sporadic in incidence. Similar disorders had not been encountered in the home or in the community. No recent acute illnesses had been observed in other members of the family, and no exposure to a recognized case of poliomyelitis was elicited in the history.

On the other hand, all of the patients became ill within the period of seasonal prevalance of poliomyelitis for the region in which they were living.

TABLE 2. SEASONAL DISTRIBUTION OF ACUTE CEREBELLAR ATAXIA ASSOCIATED WITH POLIOVIRUS INFECTION

Series	*April*	*July*	*August*	*Sept.*	*Oct.*	*Total*
Present	1		1	1	1	4
Arthius, *et al.*[8]		1	1	3	1	6
Berglund, *et al.*[1]				2		2
Total	1	1	2	6	2	12

As shown in Table 2 the onset in three cases of the present series was in August, September, and October. One child from North Carolina became ill in April; this was not an unusual time for poliomyelitis to occur in that area. In Table 2 the similar seasonal distribution of other reported cases of acute cerebellar ataxia associated with poliovirus infection is also evident.

In late August 1949 when patient B.L. (Case 1) became ill, poliomyelitis was epidemic and at the peak of incidence in Connecticut. During that year the total number of cases reported in the state was 654. Of these, 318 were classified as paralytic and, in 30 instances, death was attributed directly to the disease.* Multiple cases within a family were clinically recognized in 45 such units. It is evident, therefore, that at the time of B.L.'s illness infection with poliovirus was unusually prevalent.

As indicated in Table 3 the incidence of poliomyelitis in North Carolina was relatively high in 1956 when M. S. (Case 4) became ill and in 1959 when R. T. (Case 3) was affected. In 1956 strains of poliovirus recovered

* Data kindly supplied by Dr. Mila E. Rindge, chief, Epidemiology Section, Connecticut State Department of Health

in our laboratory from 47 patients who lived in various parts of the state were exclusively type 1. In 1959 strains of poliovirus type 1 were recovered from 81 patients and type 3 from 24. In 1960 when L. R. (Case 2) was ill the incidence of poliomyelitis was quite low and relatively few patients were studied. Strains of poliovirus type 1 were detected, however, in specimens collected during February, April, August, and October of that year.

TABLE 3. INCIDENCE OF POLIOMYELITIS IN NORTH CAROLINA**

Year	*Total*	*Number of cases* *Paralytic*	*Fatal*
1949	229		16
1950	756		24
1951	314		11
1952	538		24
1953	926		35
1954	732	297	22
1955	460	172	7
1956*	313	179	6
1957	233	51	0
1958	74	39	4
1959*	313	277	21
1960*	85	61	4

* Case of acute cerebellar ataxia in this year.
** From annual reports of Public Health Statistics, Communicable Disease Morbidity and Mortality Statistics, North Carolina State Board of Health, Epidemiology Division, Raleigh, N. C.

None of the four patients reported in this paper had received poliovirus vaccine prior to illness.

VIROLOGIC STUDIES

Specimens used in tests for the presence of virus included nose and throat swabbings, cerebrospinal fluids, and stools, and were collected during the acute stage of illness to be tested immediately or stored in a frozen state at approximately -25°C. Sera obtained during the acute and convalescent stages of illness were similarly stored prior to use.

Tests for the presence of poliovirus were carried out with specimens from each of the four patients according to techniques currently employed in the laboratory at the time.[14–18]

In the first case, a suspension of stools collected during the acute phase of illness was tested for poliovirus by intracerebral injection into a rhesus monkey. A positive result was based on the development in the monkey of muscular weakness and lesions of the central nervous system characteristic of poliomyelitis.

Specimens of nose and throat swabbings, cerebrospinal fluid, and stools from the other three patients were tested for cytopathic effect in tissue cultures of rhesus monkey kidney cells or KB epithelial cells.[17] In our experience, as reported elsewhere,[18] cultured cells of the established KB line were as sensitive as rhesus monkey kidney cells for primary isolation and propagation of poliovirus. Cultures were usually observed for a period of 10-14 days and at least two serial passages were made before a result was considered negative. Positive results were confirmed by repeated isolation from the same source. The strains of virus isolated were titrated in serial ten-fold dilutions and identified by neutralization tests in tissue cultures with type specific poliovirus antisera.* In these tests and in neutralization tests with standard strains of virus, approximately 100 TCD_{50} of virus were used. Determinations of antibody in patients' sera were made with serial two-fold dilutions within the range from 1-4 to 1-2048. Prior to testing, sera were inactivated at 56°C. for 30 minutes.

The attempts to recover poliovirus which were carried out with specimens from these patients and the results obtained are summarized in Table 4. Evidence of viral infection was detected only with fecal specimens. The agent from Case 1 which induced disease in a monkey was presumed to be a poliovirus although serologic confirmation and identification were lacking. The strains recovered from the other three patients were individually confirmed as polioviruses and identified serologically as type 1.

Neutralization tests for evidence of infection by polioviruses were carried out with sera from Case 2, 3, and 4. The results are shown in Table 4. All three patients showed serologic evidence of infection by poliovirus type 1, the agent detected in their stools during the acute stage of illness. One showed a significant rise in titer of homologous antibody above the level observed in the earliest serum available. The levels of antibody to poliovirus types 2 and 3 were not uniform in these patients.

The titers of antibody to type 3 poliovirus in sera from Case 4 were unexpectedly high and determinations were, therefore, repeated at a later date with confirmatory results as shown in parenthesis. These high levels of antibody may have resulted from the illness, diagnosed as poliomyelitis,

* Kindly provided by Dr. Herbert Wenner and the National Foundation.

TABLE 4. RESULTS OF VIRUS ISOLATION AND NEUTRALIZING ANTIBODY TESTS

Case no.	Age and Sex	Date of onset of illness	Specimen tested: Date obtained	Specimen tested: Material	Recovery and identification of poliovirus: Procedure	Recovery and identification of poliovirus: Result	Serum: Days after onset	Serum: Titer* of antibody: Type 1	Serum: Titer* of antibody: Type 2	Serum: Titer* of antibody: Type 3
1	6 yr. M	8/30/49	9/2/49	Stool	Intracerebral injection into rhesus monkey	Poliovirus				
2	20 mo. F	4/4/60	4/11/60	Throat swab	Culture in KB cells	Negative	7	64	4	4
			4/11/60	Nose swab	"	Negative	32	256	4	4
			4/11/60	CSF	"	Negative				
			4/12/60	Stool	"	Poliovirus Type I				
			4/13/60	Stool	"	"				
			4/15/60	Stool	"	"				
3	6 yr. F	10/21/59	10/25/59	Nose and throat swab	Culture in KB cells	Negative	5	64	8	<4
			10/27/59	CSF	"	Negative	36	64	8	<4
			10/27/59	Stool	"	Poliovirus Type I				
4	22 yr. F	9/21/56	9/25/56	Nose and throat swab	Culture in rhesus monkey kidney cells	Negative	6	32	8	256(512)**
			9/25/56	CSF	"	Negative	13	32	8	1024(256)
			9/28/56	CSF	"	Negative	41	32	16	2048(1024)
			9/28/56	Stool	"	Poliovirus Type I				

* Titer expressed as reciprocal of serum dilution.
** ( ) Results of titration repeated at a later date.

which this patient had experienced four years earlier. The increase in titer of type 3 antibody is of questionable significance but may possibly represent an anamnestic response to the recent type 1 infection.

With specimens from these patients various attempts were made in suckling mice and tissue cultures to recover additional viral agents and to demonstrate serologic evidence of infection by other viruses. The results were negative.

DISCUSSION

It is evident that acute cerebellar ataxia exists as a well defined clinical entity by virtue of arbitrary criteria to which an individual case may, or may almost conform. Strict classification invites the exceptions of biologic variation, as exemplified in the present series of cases. Diagnosis determined exclusively by such classification is of limited value, however, and may be misleading as a basis for prognosis. Nevertheless, it serves a useful purpose as a stimulus to seek more precise identification of etiology and a clearer understanding of the altered physiologic mechanisms involved.

As mentioned previously, cerebellar ataxia may reflect a number of different clinical conditions and occurs acutely either independently or in association with other manifestations of disease. The appearance of acute ataxia in the course of varicella is sufficiently frequent to be well recognized and cases associated with varicella can be readily related to outbreaks of this disease. Whether acute cerebellar ataxia can also be attributed to infection by one or several other different viruses is a question which has been raised frequently but not fully answered. Clinical observers have postulated that poliovirus may be responsible in some cases.[3,5,11–13] Lasater sought to support this view with evidence of high levels of neutralizing antibody to type 3 poliovirus in two of his cases;[13] rising titers were not demonstrated, however, nor were strains of virus isolated. Prominent cerebellar signs and symptoms have been observed in the course of infectious mononucleosis[19] and with ECHO virus, type 9 infection.[9,10] Acute cerebellar ataxia has also been noted in a patient presumed to have had infection with influenza A virus on the basis of an associated rise in titer of antibody to this agent.[20]

The contention that poliovirus may play an etiologic role in some cases of acute cerebellar ataxia is favored by clinical evidence of ataxia in acute poliomyelitis, by pathologic evidence of cerebellar involvement in fatal human cases of poliomyelitis as well as in experimental animals and, more recently, by the demonstration of direct association of poliovirus infection with the syndrome as exemplified in this study.

Since 1898 when Medin reported that five of his cases of poliomyelitis had exhibited acute, transitory ataxia,[21] clinicians have recognized that cerebellar signs can and do occur in patients with this disease.[22–25] In a comprehensive study of poliomyelitis Fanconi *et al.*[23] reported the occurrence of ataxia in 12 of 37 cases of polioencephalitis but mentioned none with ataxia alone. It is not clear whether this omission was because cases of acute cerebellar ataxia were not observed or because they were categorically excluded from consideration. Ford has stated recently that "acute cerebellar ataxia without any typical manifestations of poliomyelitis is probably never a result of Heine-Medin Disease."[24] Debré and Thieffry have expressed a similar opinion.[26] These views, however, appear to be inconsistent with the concept, clearly postulated by Wickman[25] and since then extensively confirmed, that poliovirus infection may be reflected by a wide and varied spectrum of clinical manifestations. If ataxia can occur during infection with poliovirus in cases with paralysis why not in cases similarly infected but without paralysis?

From microscopic examinations of the central nervous system in fatal human cases of poliomyelitis and in experimentally infected animals, it is evident that the cerebellum is frequently affected and often severely damaged.[22, 25, 27, 28] In a survey of 24 fatal cases of human poliomyelitis Bodian assessed the intensity of pathologic damage in motor centers.[27] He found that damage in the cerebellar roof nuclei occured in all cases and was exceeded in severity only by that in the vestibular nuclei and the reticular formation. He noted wide variation in intensity of lesions in motor centers in both human and experimental poliomyelitis. Similarly located lesions of moderate intensity were also observed in symptomless or nonparalytic experimental infections. Comparable observations of nonparalytic poliomyelitis in man are relatively few but the findings are similar. Therefore, on the basis of histopathologic evidence it is not surprising that cerebellar signs occur in patients infected with poliovirus—indeed from the frequency and distribution of lesions it is difficult to understand why in clinical experience these signs are not more common.

Direct laboratory evidence of poliovirus infection in cases of acute cerebellar ataxia has been presented in some detail by investigators in Stockholm and Paris and briefly in a recent abstract from Puerto Rico.

Berglund *et al.* in Stockholm described two children, aged 3½ and 5 years, with marked cerebellar symptoms, from whose stools type 1 poliovirus was isolated.[1] They had little or no prodromal signs, no fever, no nuchal rigidity, and lumbar punctures revealed normal cerebrospinal fluids containing 2 and 7 leukocytes/cmm. respectively. Both patients recovered

completely. Rydenstam, one of the original co-authors, again reported these two children in an article concerning atypical manifestations of polioencephalitis.[29] These cases, like the first two in the present series, conform to a strict definition of the syndrome.

In 1960 Arthius *et al.* in Paris reviewed their experience with six cases of acute cerebella ataxia, associated with the isolation of poliovirus type 1 from the stools of five and of type 3 from the sixth.[8] Some of these patients had been mentioned in earlier articles.[30] Four were in the 2½ to 4 year range; two were 9 and 13 years old respectively. In these cases prodromal signs were present, all but one was significantly febrile, four of the six showed other neurological deficits (dysphagia, unilateral facial weakness, or difficulty with urination), and all but one had a pleocytosis of 52 to 143 leukocytes/cmm. in the cerebrospinal fluid. The exceptional one had 9 cells/cmm.

The authors concluded that acute cerebellar ataxia associated with poliovirus infection can be differentiated from a purer form of this syndrome of unknown etiology by the presence of prodromal symptoms typical of poliomyelitis, fever, nuchal rigidity, and pleocytosis of the spinal fluid.[9] This conclusion, however, is not supported by the observations of Bergland *et al.*[1] nor by our experience with cases 1 and 2.

Méndez-Casion *et al.* reported experience with acute cerebellar ataxia in eleven children under five years of age seen during a period of 3½ months when poliomyelitis (type 1) was epidemic in Puerto Rico.[31] Two of the children showed cerebellar signs only; eight also had facial paralysis and in one transient ptosis of the left eyelid was observed. Laboratory evidence to be reported in detail later was said to support an association with infection by poliovirus type 1.

In the present series, cases 1, 2, and 4, particularly 1 and 2 were remarkable for their unusual ocular movements. In cases 1 and 2 these movements were coarse, jerky and unpredictable in pattern. They were conjugate but completely irregular in frequency and amplitude, as well as in direction, although the horizontal plane predominated. They were increased by movement of the head or trunk or by superimposed volitional movements of the eyes alone. Apparently Berglund's two patients[1] and cases 3 and 4 of Arthius *et al.*[8] showed a similar picture. This phenomenon, which is unlike classical nystagmus, appears to have been described first in Warsaw by Orzechowski, who coined for these myoclonic-like movements the term "opsoclonus."[32] He stated that they give an "impression of great agitation of the eyeballs; chaotic, variable, and almost impossible to analyse." Marmion and Sandilands apparently observed something similar

in a 2 11/12 year old boy during the 1947 poliomyelitis epidemic in England,[33] although Cogan would prefer to define what they described as "ocular dysmetria" or "ocular flutter" since it became worse just prior to fixation.[34] Strickland also noted abnormal ocular movements that were enhanced by an attempt at fixation in three children who were seen during the same epidemic.[35] Since the abnormal movements in his cases were limited to the horizontal plane they fit far better Cogan's definition of "ocular dysmetria." The ocular movements in case 4 of the present series would appear not to be typical of any of these conditions, but are best described as a form of nystagmus (horizontal and slightly rotary on looking to the right, horizontal on looking to the left, and horizontal to the right on looking upward). In this regard, Murray and Walsh have stated that they have found nystagmus, usually horizontal but occasionally vertical or rotatory, to be the commonest ocular complication of poliomyelitis.[36]

The six cases reported to date of poliovirus infection associated with cerebellar signs and so-called "opsoclonus" each had as the other dominant feature marked intention tremor involving the entire body (cases 1 and 2 of the present series, Berglund's two cases,[1] and Arthius' cases 3 and 4[8]). Arthius *et al.* have described these eye movements as "a true ocular tremor" ("un véritable tremblement oculaire"), "so spectacular that it immediately permits one to recognize the illness."[8]

Although the syndrome of acute cerebellar ataxia has often been regarded as completely reversible, Weiss and Carter have clearly shown that frequently this is not true.[7] They found the outlook for complete recovery to be far less promising when the cerebellar signs were severe, especially when associated with tremor of the head and trunk and with abnormal eye movements. Both of these features were prominent in cases 1 and 2 of this report. It is not surprising, therefore, that ataxia and intention tremor were still demonstrable in case 2, 15 months after the onset of her illness.

Since the combination of abnormal ocular movements and tremor of the head and trunk has been conspicuous among the reported cases of acute cerebellar ataxia associated with poliovirus infection, the prognosis for complete recovery in these cases may not be assured. On the other hand all such patients reported to date with the exception of case 2 in this series have apparently recovered completely.

SUMMARY

Poliovirus was recovered from the stools of four patients with acute cerebellar ataxia. In three the virus was identified as type 1 and neutralizing antibodies to this agent were demonstrated.

Three of the patients were children 6 years of age or younger; one was a woman of 22 years. Two of the children had acute ataxia unassociated with prodromal symptoms, muscular weakness, nuchal rigidity, abnormalities of cerebrospinal fluid or significant fever. Three of the patients exhibited unusual ocular movements, in two characteristic of "opsoclonus." One of the children failed to recover completely.

The significance of acute cerebellar ataxia as a clinical entity and its association with poliovirus infection has been discussed. Evidence, based on clinical, epidemiologic, pathologic, and virologic observations, indicates that the association of poliovirus infection with the clinical manifestations in the reported cases was probably not fortuitous, and that polioviruses should be included among the viruses considered capable of causing this syndrome.

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GILBERT DALLDORF*

Sloan-Kettering Institute for Cancer Research, New York

A GREAT EXPERIMENT

"It is good thus to try in imagination to give any one species an advantage over another. Probably in no single instance should we know what to do. This ought to convince us of our ignorance on the mutual relations of all organic beings: a conviction as necessary, as it is difficult to acquire."

—*Charles Darwin.*

It may be characteristic of our age that so tremendous an experiment as the infection of seventy-seven million Russians with three avirulent strains of poliomyelitis virus was undertaken as with the sweep of a hand. Perhaps only a commissar's hand could do it. But much may be due to the arrogant confidence of twentieth century man and his habitual disregard of the limitations of his knowledge.

Mass use of the oral vaccine followed careful small trials which firmly established that the viruses used did not of themselves cause disease. And the result of the mass application seems to have been an outstanding success. Poliomyelitis dwindled to a fraction of the number of cases reported in the preceding years. Indeed, these trials have emboldened some experts to confidently predict the *eradication* of the poliomyelitis viruses. Time alone can tell whether that is feasible or even possible. Meanwhile it seems of some interest to consider certain biological phenomena that will presumably affect the final result and that characterize the undertaking as a great experiment.

The prevailing view seems to be that truly comprehensive immunization of a population with the vaccine viruses, feasible because of the ease of administration and economy, may provide a degree of active immunization and refractoriness to reinfection that will eliminate all possible hosts of the epidemic virus strains. Such an undertaking can doubtless be more easily carried out in a rigidly managed society but it is, of course, quite likely that universal immunization would not be required, that a threshold would earlier be reached at which the propagation of the field strains of viruses would cease. The extent of immunization necessary for control is an important value still to be determined.

A greater effect would follow if the vaccine virus strains were capable of suppressing, of successfully competing with, the field strains. Under such

*Chief of the Division of Experimental Pathology of the S.K.I.

conditions it would be necessary only to seed the attenuated strains into a population and await their spread. This seems unlikely with the present strains which appear less capable of spread and prolonged excretion than the field strains. Doubtless other characteristics may play a part. Nevertheless, we have no reason at present to expect that control will be possible through the mere introduction of the modified viruses. Moreover there is considerable evidence that the attenuated viruses, if they were propagated through a susceptible population, might eventually acquire virulence and change toward their original form.

It therefore seems much more likely that complete control or eradication would be accomplished, if at all, through universal immunization much as smallpox has been eradicated. In practice, repeated doses may be required, since a present disadvantage of the poliomyelitis vaccines, in comparison with vaccinia, is our inability to recognize successful vaccination short of laboratory tests while the immune status to vaccinia may be easily established by inspection.

Other differences between these two live virus vaccines and the diseases they prevent are still more significant. The most conspicuous difference that makes the Russian trials an important experiment and introduces endless biological complexities is that there are three polioviruses and some fifty cousins of them showing various degrees of likeness. The mass use of the vaccine strains, therefore, provides an opportunity to observe the ecological effects of a drastic change in the intestinal virus flora, a feature wholly lacking in the case of vaccinia.

Ecological problems have been neglected in the animal virus field, despite much suggestive evidence that they may eventually prove of considerable importance. Animal virologists somewhat belatedly (plant virologists were already fully familiar with these phenomena) recognized that certain viruses interfere with others. Attention was drawn to such reactions twenty-five years ago by two independent studies in one of which yellow fever[1] and in the other experimental poliomyelitis[2] were shown to be influenced by particular concurrent infections. Initially, attention was attracted to the sparing effect which one infection had on the disease caused by the second. This was quickly extended to susceptibility to infection as well as resistance to the disease. The phenomenon is now generally known as *interference*. It has been studied chiefly in terms of experimental diseases or cultivated cells. Its epidemiologic significance has been only superficially explored.

The recognition of three distinct types of poliovirus was followed by the discovery of other enteric viruses having similar properties. The list of these now numbers nearly sixty small, ether resistant human pathogens (or potential pathogens) and many additional ones that are native to

lower animals. Many of the former are known to be quite similar to the polioviruses including when and where they may be found. They cohabit. A number of them interfere with or are interfered with by the polioviruses. Others may infect simultaneously with the polioviruses and may possibly contribute to the severity of the resultant disease. It is in terms of this complex ecological relationship that the wholesale use of vaccine viruses may profitably be considered.

Antagonisms between the polioviruses themselves are well known. Presumably the common tendency of one type to predominate during epidemics is an expression of that antagonism. It became evident during the early trials of the vaccine viruses when it was observed that simultaneous ingestion of all three types sometimes led to infection with merely one. It is therefore recommended that the three types be given independently at intervals of several weeks. This routine has been somewhat modified in Russia but not in principle. The Russian practice is to combine two types in the second dose and three in the third in the expectation of benefiting individuals who may have escaped infection with the repeated type during the first exposure.

The extent to which interferences between the vaccine viruses and their cousins may determine the effectiveness of vaccination has been evident in many trials, especially in areas and at times when the cousins were prevalent. An extreme situation occurred among a Mexican population of low economic status.[3] One of the groups included in that study was known to be excreting non-polioviruses. Only three of the thirty-three who received oral vaccine became infected and only fourteen of the forty-seven studied developed antibodies to the type of vaccine virus administered. In consequence of such observations, vaccination with the attenuated viruses is only undertaken during seasons of low incidence of infection with enteric viruses, although it may be that viruses will be found and dosages established for them that would make it possible to dispossess the interfering agents and successfully immunize under such circumstances. But these are relatively trivial, practical problems implicit in the use of the live virus vaccines. More interesting issues lie beyond them.

The antagonisms between enteric viruses are presumably quite varied. There is a pronounced interference between Group B Coxsackie and type 2 poliovirus infections in mice,[4] and the influence of Group B infections persists for a surprisingly long time.[5] Considerable evidence has been assembled to show that Bornholm disease or epidemic pleurodynia (which is caused by Group B viruses) suppresses paralytic poliomyelitis in man.[6] These two diseases characteristically have a reciprocal relationship when judged by numbers of reported cases; because both are highly characteristic

clinically these data seem significant. The Danish records provide the best raw data since both were reportable diseases in Denmark for many years and Danish physicians were expert in their recognition. During the twenty-five years for which records exist, epidemics of paralytic poliomyelitis and epidemic pleurodynia never occurred simultaneously. In seven of the years both were infrequent, in eighteen of the years one or the other was epidemic.[7] Interestingly, it was a Dane who first called attention to the epidemiologic similarities between the two.[8]

Striking interferences have been demonstrated experimentally between Group A Coxsackie viruses.[9] The two most completely studied (A-10 and A-14) are known to be quite similar to the polioviruses. Strains of A-14 may cause, in mice, a disease indistinguishable from poliomyelitis and both have been associated with paralytic disease in man. Relatively few of the Coxsackie viruses and none of the ECHO viruses have been methodically examined to determine the range and degree of whatever interfering effects they may have on the family of enteroviruses. However, enough is known to justify the suspicion that great variations in their interrelationships will be found to exist.

The massive dissemination of vaccine virus strains, in view of these facts, may be of special interest on three counts. It seems evident that the immediate effect of the rapid infection of millions of subjects may differ considerably from the late effect. One would expect that the introduction of live enteric viruses to a large part of the population would interfere not only with the poliovirus, but certain other enteric viruses as well, and that the suppressed viruses might in some cases be displaced or excluded. While the vaccine viruses may be incapable of competitively displacing the native strains of polioviruses, they may exclude other enteric viruses and establish a changed pattern of infection which could conceivably persist.

It is also possible that the elimination of the polioviruses through sustained widespread immunization would be reflected in a greater prevalence of whatever enteric viruses they may have suppressed in the past. The Group B Coxsackie viruses would seem likely candidates and it will be of considerable interest to observe, once the immediate effects of vaccination have subsided, whether epidemic pleurodynia becomes more common in Russia. During a recent discussion with M. P. Chumakov, who has directed the Russian program, I was told of outbreaks of paralytic disease which he had established as being caused by certain Group A Coxsackie viruses including Type A-7 which, when it was first identified in Russia, was considered to be a new type of poliovirus. There are substantial reasons, indeed, for considering several of the Group A strains to be much more like the three accepted types of polioviruses than most of the enteroviruses. The

impression I gained from the discussion was that most of the paralytic "poliomyelitis" that occurred in Russia during 1960 following the large scale immunization was due to Coxsackie viruses rather than polioviruses. Whether this is the harbinger of the emergence of certain of these viruses as important epidemic strains is not yet known. Ecologic changes have rarely been predictable. We can expect to do no better than explain whatever changes occur after they take place.

Underlying all these considerations are problems of viral genetics which are so much a part of species competition. Because of the number and variety of the enteric viruses and their frequent coexistence, the possibilities of change are of a high order indeed, and if these prospects be added to what is already recognized as a current feature of enteric virus infections, namely their increasing importance in consequence of changing hygienic practices, we should prepare ourselves for further alterations in the nature and behavior of the numerous, worldwide little viruses.

In all of these ways the massive, abrupt, and nationwide introduction of experimentally modified virus strains may be presumed to be a huge experiment, unique in its size and boldness and rich in opportunities for the study and understanding of the ecologic relationships of human viruses. One would hope that every effort will be made to follow such experiments closely, to observe and record with an open mind. The opportunity is a challenging one.

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JOHN F. ENDERS*

Department of Bacteriology and Immunology, Harvard Medical School, Boston

VACCINATION AGAINST MEASLES: FRANCIS HOME REDIVIVUS

FOREWORD

This paper is an attempt to review in the light of earlier research certain recent work on measles virus with emphasis on the development of a living attenuated vaccine against the disease. It was presented as a Cameron Prize Lecture before the Faculty of Medicine, Edinburgh University, 16 May 1961. Since it has not been submitted for publication the author decided it might be appropriate to offer it as his contribution to this collection of papers that jointly testify to the great respect and affection which all of his colleagues, pupils, and friends have for John Paul.

Although written for another occasion it seemed fitting for several reasons to include it here. There is, among John Paul's earliest professional communications, one devoted to a pathological study of measles conjunctivitis. Throughout the years as epidemiologist and distinguished student of Preventive Medicine he has continued to maintain his interest in this ubiquitous and fascinating disease. Recently in John Paul's laboratory significant additions to our knowledge of measles have been made by several members of his staff whose investigations he warmly encouraged. Finally, as director and afterwards member of the Commission on Viral Diseases of the Armed Forces Epidemiological Board he has, by his helpful enthusiasm and wise counsel, contributed substantially to the studies in our own laboratory which are described in this paper.

INTRODUCTION

The problem of vaccination against measles is an old one, and only now may the solution be at hand. Its antiquity is accented by the fact that the first attempt to immunize against measles preceded by nearly half a century Jenner's discovery of smallpox vaccine. This first attempt was made in 1758 by Francis Home of Edinburgh. Home was then a Fellow of the Royal College of Physicians and, to judge by his many excellent

*Chief, Research Division of Infectious Diseases, The Children's Hospital Medical Center, and Professor of Bacteriology and Immunology at the Children's Hospital, Harvard Medical School.

case reports, an active practitioner in the City and in the Infirmary. Later he became Regius Physician and Professor of Materia Medica in the University.

In reading Home's prototypic experiments as well as some of his other medical writing. I have been impressed with the keenness of his powers of observation, the logic and modernity of his thinking, his experimental approach to clinical problems, and his healthy scepticism. These characteristics reveal the father of inoculation against measles as a somewhat neglected forerunner of the great age of experimental medicine that was to come in the next century.

Measles in Home's time appears to have presented a far more serious problem than it does nowadays, at least in the so-called "well developed" countries. In other parts of the world, however, as I shall later reemphasize it still frequently takes the form of severe illness with a mortality that is far from negligible. The gravity of the disease in mid-eighteenth century Scotland is well exemplified by Home's statement at the beginning of his book entitled "Medical Facts and Experiments" in which he described his work on vaccination. Here he remarks, in what to us today appears to be a monstrous understatement, that in the epidemic which occurred in Edinburgh about the beginning of December, 1758 the measles were "in general of a mild sort; and not about the twelfth part died of those who were attacked". Further on he implies that in former outbreaks the incidence of serious complications, especially that of pneumonia, as well as the mortality was much higher.

This disturbing situation led him to attempt inoculation of "morbilous matter" by a parenteral route in the hope, as he puts it, of rendering "the disease more mild and safe in the same way as the Turks have taught us to mitigate the smallpox". Home's theoretical basis for this procedure appears eminently logical. "I suspected strongly", he wrote, "that the cough, often so harassing, even in the mildest kind, was produced by receiving the infection mostly by the lungs; and I hoped that this symptom would abate considerably if I could find a method of communicating the infection by the skin alone".

At the outset Home was confronted with a major difficulty; for, unlike smallpox there was, as he says, "no matter to be had from the measles" that appeared suitable for inoculation. He goes on: "A woolen glove taken from the arm of a measly patient would not answer my purpose, as part of the infection might be drawn in by the lungs. I could not find a sufficient quantity of scaly matter, after the measles were dried, to serve my purpose". Characteristic of his intuitive correctness of thought was the way he

"o'er leapt" this hurdle: "I then", he declares, "applied directly to the magazine of all epidemic diseases, the blood". Not only in the choice of blood as inoculum but also in that of the optimal time for taking it he displayed much discernment: "As the measly matter behoved to be but a small proportion of the whole mass, I chused to make use of the blood when it contained the morbilic matter in the highest state of acrimony". He reasoned that the time of the "highest state of acrimony" or, as we should say, virulence, was at the peak of the fever, shortly after the eruption appeared. Experiments of the present century have justified Home's choice since they showed not only that viremia regularly occurs but that the virus present in the blood at the onset of the eruption soon thereafter disappears from the circulation. Noteworthy also are his conjectures that the amount of the virus in the blood is small and, as he points out later, may rapidly lose its infectivity if the blood is too long preserved before inoculation. These enlightened guesses have lately received ample experimental confirmation by investigators such as Ruckle and Rogers and ourselves, using the tissue culture method which I shall soon mention.

Home described the results of inoculation of measles blood in a group of twelve children. In ten of them an eruption that he considered characteristic of the disease was noted following a rather regular interval. Two of the oldest children, one of whom gave a history of measles, failed to present an exanthem. Rash, when it occurred, was usually preceded by fever, signs of conjunctivitis and coughing. These manifestations appeared most frequently six to seven days after inoculation and usually preceded the rash by one to three days. That he "might see the difference of the disease when it is communicated by the lungs alone and when by the skin alone" Home inoculated three other children with blood or nasal discharges from measles patients. None of these three subsequently came down with the disease.

A number of subsequent workers failed to confirm his observations with measles blood while a few reported success. Prophylactic inoculation was never exploited on a large scale presumably because of these negative trials and because of the difficulty of obtaining material for inoculation at a convenient time. Difficulty of preserving the infectivity of the inoculum also probably spoke against its adoption. Finally Hektoen, in 1905, in a detailed and critical review of Home's results, seemed to give the coup de grace to the claim that he had achieved the experimental transmission of measles.

Hektoen sweepingly concluded that probably not one of Home's cases developed measles as a result of inoculation because the incubation period

fell short of that in the natural disease, being in no case longer than 10 days, and because Home's failed to exclude the possibility of natural exposure prior to inoculation. The force of Hektoen's objection that the incubation period was too short to be compatible with measles is, I believe, largely dissipated by our own observations which I shall subsequently present, observations on the accelerated development of signs of modified measles following administration of an attenuated measles virus. Hektoen's second objection cannot be so readily met since it must be admitted that chance exposure of some of the subjects may have occurred. Indeed, Home himself was quite aware of this possibility. But as he takes pains to point out, there were at least in two instances in which inoculations were made at the end of August when there were no cases of natural measles in the community. Furthermore, it would seem unlikely that so large a proportion of the children would have developed measles after approximately the same interval following inoculation had previous exposure been responsible.

I suggest, therefore, that the laurels, of which Home seems to have been unjustly denied, should be, in the light of recent findings, restored to this pioneer in the experimental study of measles. In any event, recent investigations which I shall summarize will serve to show that his basic ideas concerning prophylaxis in this disease still live on.

THE VIRUS OF MEASLES

Historical. Any attempt to develop a safe and effective vaccine against an infectious disease must be preceded by identification of the etiologic agent, its isolation in a pure condition or in a state free of other pathogenic microorganisms, its reproduction in sufficient quantities, and its preservation in an antigenically stable form. To those must be added objective tests for resistance of susceptibility and methods for assay of antigenic potency of the vaccine. Home's attempt to inoculate against measles proved abortive because in his time none of these technical requirements could be adequately met. Indeed in the case of measles virus it is only within the past seven years that reliable and effective procedures for meeting them have been developed.

This tardiness is not attributable to lack of effort, since during the preceding fifty years of this century numerous investigators sought to discover susceptible animal hosts, and to propagate the virus in tissue cultures or in the developing chick embryo. The results of these numerous investigations were relatively meager, but certain valuable contributions were made. Indian monkeys inoculated with materials from measles patients

were found by Goldberger and Anderson to develop a mild form of the disease. This observation was confirmed, although certain investigators noted that frequently individual monkeys failed to develop overt signs of infection. This variation in susceptibility rendered the experimental use of these animals difficult or unsatisfactory. Numerous accounts of successful inoculation of other species, such as guinea pigs, rabbits and mice, appeared from time to time but none received general confirmation. Similarly several authors described the propagation of the virus in chick embryos and in chick cell tissue cultures. But again consistent confirmation was lacking. Perhaps the most notable of these studies in which chick embryos were used are those of Rake, Shaffer and Stokes, who in about 1940 inoculated monkeys and a large group of children with materials from serial egg passages that were initiated with infected blood or throat washings from measles patients. Signs of modified disease were observed in a small proportion of the children, but on subsequent exposure to measles little or no protection was afforded and the investigations were not extended. Rake and his co-workers were handicapped by the variable response of the monkey, and lack of any means for the detection and measurement of specific measles antibody whereby they might determine the antigenic potency of the vaccine or distinguish between susceptible and resistant animals or human beings.

Because of these many equivocal results it became increasingly apparent that to achieve further progress two things were needed: first, a simple culture system which would be affected by the virus in such a way as to make it easily apparent that viral multiplication had occurred within the system itself; second, experimental animals of uniform susceptibility to the virus. Given these two essential tools, one felt confident that reliable techniques could be readily developed for isolating the virus, studying changes in its virulence, and producing it in quantity. Procedures would also be provided for the detection and assay of antibodies and for determining the potency of any vaccines that might be developed.

Isolation in Tissue Culture. It seemed that the tissue culture techniques which Weller, Robbins, and I had successfully used in the propagation of the polioviruses might at least provide us with one of these tools. Accordingly, in 1954, with Dr. Thomas Peebles, blood and throat washings taken in the early exanthematous phase from typical cases of measles were added to roller tube cultures of normal human kidney tissue. After a considerably longer incubation period than is characteristic of poliovirus

we saw changes in the new outgrowth of renal epithelial cells which were quite unfamiliar and therefore exciting.

These changes occurred focally and consisted of loss of cell boundaries with consequent mingling of cytoplasm to form an extensive mass in which numerous nuclei lay embedded. When stained, affected areas closely resembled syncytia or multi-nucleated giant cells. In nearly every nucleus within the syncytium large, intensely staining eosinophilic inclusion bodies were seen, surrounded by a clear halo. In the syncytial cytoplasm eosinophilic bodies of irregular size and shape were frequent, but not invariable, features. These cytopathic effects proved to be reproducible in series when the fluid from an infected culture was transferred to a fresh preparation. The concentration in the culture fluid of the transmissible agent thus revealed could now be easily determined by testing increasing dilutions of the fluid for their capacity to induce these changes. Filtration through bacteria-retaining membranes and failure to demonstrate bacteria in infected fluids served to provide definite evidence for the viral nature of the agent we had cultivated. These observations have been repeatedly confirmed by other workers.

Serological Identification. Evidence strongly suggesting that this agent was in fact the virus of measles was obtained by showing that the distinctive cytopathic changes failed to appear when the serum from patients convalescent from measles was mixed with the virus-containing tissue culture fluid and introduced into fresh cultures. In contrast sera taken in the early acute phase exhibited no such suppressive or neutralizing effect. The virus-neutralizing effect of convalescent phase sera was thus shown to depend on a specific antibody that develops during the course of measles. Furthermore, we found that fluids from infected cultures contained an antigen which fixed complement with convalescent but not with acute phase measles serum. Very recently Chany in France and Rosen in the United States have shown that measles virus hemagglutinates rhesus monkey red cells and that this hemagglutination is specifically inhibited by convalescent measles serum.

Effect of Virus on Monkeys. Additional assurance that we were dealing with the virus of measles was obtained when we inoculated fluids from infected cultures into susceptible monkeys. Before these inoculations were done, since we now had the techniques available, and since we had in mind the irregular response of these animals noted by earlier workers, we tested the sera of rhesus and cynomolgous monkeys for the presence of measles antibodies. The animals first selected had been held for considerable periods of time in three different laboratories. Virus neutralizing and complement fixing antibodies were found in all eight of the cynomolgous

and in thirteen of sixteen of the rhesus monkeys tested. Frequently the concentration of antibody was high. When a few of these monkeys were inoculated with the tissue culture virus they failed to present any signs of infection. Here apparently we had a satisfactory explanation for the variation in susceptibility of individual monkeys.

To explain these observations it occurred to us that animals with antibodies might have become infected with measles virus or a closely related agent following their capture and confinement in the laboratory. To test this hypothesis we procured, with the assistance of the United States Armed Forces, a number of cynomolgous monkeys that had just previously been captured in the Philippines. No measles antibodies were detected among the first ten animals examined. Subsequently we tested approximately fifty additional cynomolgous monkeys that were shipped to us promptly after capture in the Philippines or Malaya and found them to be quite free of antibody. Comparable results have now been obtained by several other workers.

When monkeys exhibiting negative complement fixation or neutralization tests were inoculated with culture fluids containing the agent one or more manifestations of infection became apparent. These consisted of viremia discernible about the 4th or 5th day which persisted for several days thereafter; moderate leucopenia lasting from about the 7th to the 11th day and a macular measles-like rash on face, trunk, and abdomen. In all animals antibodies specific for the virus began to appear in the blood about the 15th day. Of the various signs, viremia and the emergence of antibody proved to be the most constant and reliable.

Taken as a whole these observations provided the basis for reinvestigation of various aspects of the disease and its causative agent including the problem of specific prophylaxis.

Other Relevant Properties. i) Antigenicity. Since these tools became available a great deal has been learned through the efforts of a number of investigators in Europe, Japan, and America about the nature and properties of measles virus. As background for the studies on vaccination to be reviewed, I shall summarize rapidly a relevant portion of this new information.

Perhaps most important is the demonstration of the high antigenic potency of the virus when it is introduced into the body in an infective state. Serological analyses carried out with the new techniques by Black have demonstrated the presence of neutralizing and complement fixing antibodies in the serum of over 90 per cent of older persons in an urban population in Connecticut. Titers of these antibodies ranged from about

1:16 to 1:64 or higher in more than one half of Black's subjects. These concentrations are not greatly inferior to those attained soon after an attack of measles. The antigenic efficiency of live virus is also emphasized by the rapid mobilization of antibody following infection. Bech, for example, in a recent analysis of a large number of sera from cases in a measles outbreak that occurred in Greenland detected antibodies in a significant proportion as early as 48 hours after onset of rash and in all sera examined within 5 days thereafter. In a subsequent study Bech found that in 90 per cent of persons examined little change in antibody concentration occurred between the 1st and 5th years after an attack.

These observations testifying to the solid, lasting immunity resulting from natural infection encourage the belief that a vaccine composed of an attenuated live virus might likewise induce a satisfactory state of resistance.

Concerning the antigenicity of measles virus after inactivation by heat or chemicals there are so far few published data. However, these are sufficient to indicate that after inactivation by formaldehyde, or by gentle heating, antigenicity may be retained despite repeated injections of the inactivated material which are required to achieve antibody levels approaching those established by infection. Very little is known as yet about the persistence of antibody after immunization with inactivated virus or about its capacity to protect upon exposure to measles.

ii) *Antigenic Homogeneity.* Fortunately for those contemplating vaccination against this disease, it now appears that all strains of the virus are probably antigenically homogeneous. No differences in specificity have been distinguished between viruses isolated in England, Denmark, Japan, and the United States. Moreover, several strains recovered in successive years from cases in the area of Boston, Massachusetts have, in our hands, proved indistinguishable in respect to antigenic composition. It is, of course, possible that strains may be discovered that are not antigenically homogeneous with those known at present. To me, however, the existence of such heterologous strains is unlikely, since one would expect the incidence of second attacks of measles would be higher if different antigenic types existed in nature. This reasoning is supported by the situation in the case of mumps where second attacks are also a rarity and where over a period of twenty years no significant antigenic differences have been noted between the numerous strains of virus that have been studied.

iii) *Capacity to Multiply in Cells of Various Species and Types.* It has been shown in many recent experiments that measles virus, once it has been isolated and grown for a number of generations in human or monkey cell cultures, can be adapted more or less readily to other cell systems such as bovine kidney cells and a variety of continuous or laboratory-maintained lines of human and monkey cells. The Edmonston strain has also been adapted to chick embryo cells. Adaptation to chick cells is of much significance for the problem of vaccination and I shall refer to it again in more detail. Certain of these systems offer advantages over others for particular purposes. Because of the known malignant character of some and the possible malignant character of others it has not been proposed to use any of the available continuous cell lines for propagation of virus to be used in the preparation of vaccines. For this purpose only chick embryo, primary human amnion, and canine renal cell cultures have so far been given serious consideration.

iv) *Stability.* In relation to vaccination with an attenuated virus such as we have developed, the stability of the agent on storage under various conditions is obviously of much practical importance. It has been determined that in complex fluid media such as are used to nourish tissue cultures measles virus rapidly loses its infectivity at 37°C. Happily, at icebox temperatures activity is preserved for several weeks and, as we found in our laboratory, at the temperature provided by solid CO_2, it may be preserved at least for four years. It has also been demonstrated in the laboratories of several pharmaceutical manufacturers in the United States that when suspended in a suitable chemical milieu the virus can be dried from the frozen state with only moderate loss of infectivity. When once frozen and dried, it may be kept in the icebox for many months with little or no decrease in activity. Thus the stability of the measles virus, although far less than that of certain others such as poliovirus, is, under appropriate conditions, sufficient to permit manufacture and distribution of an attenuated virus vaccine.

v) *Yield of Virus in Tissue Culture.* In the future if immunization against measles should be practiced as routine, large quantities of vaccine would be required. Therefore, the characteristics of the growth of measles virus in various cell systems also becomes a matter of practical interest. As I have already remarked, this agent grows at a slower rate in comparison with certain other viruses. In unpublished experiments done several years ago employing human renal cell cultures, we found little evidence of virus multiplication as judged by assay of the nutrient fluid until about the 6th day. Maximum yields were not obtained until about the 9th day.

In chick cell cultures multiplication of the attenuated variant that we have tested as vaccine follows an essentially similar pattern.

The concentration of virus attained in the fluid phase does not usually exceed 100,000 tissue culture infective doses per ml. The quantity of virus within the cells is about 10 times greater. Once viral reproduction has reached a maximum it remains at a steady rate for days or even weeks in primary cell cultures. At this maximum the rate of reproduction is exceedingly rapid as determined by Black and by ourselves in unpublished experiments. This fact gives ample assurance that measles virus can be easily and economically produced in large quantities, since successive harvests of infected fluids can be made from a single set of cultures.

vi) *Cross Reactions with CDV and Rinderpest Viruses.* My final remarks on the properties of measles virus concern the evidence for antigenic relationships between this agent and two viruses causing disease in lower animals, namely canine distemper and rinderpest virus. As long ago as 1928 Bryan postulated, on the ground of pathological and clinical similarities, a relationship between the agents of measles and distemper. Pinkerton and Adams in the United States subsequently embraced this hypothesis and supported it with additional data of the same sort. Karzon in 1955 showed that a large proportion of adolescents have neutralizing antibodies for canine distemper virus. The possibility was then raised that these antibodies develop as a result of infection with measles virus. Subsequently several workers have noted an increase in distemper antibodies following infection with measles virus. In contrast, it has proved impossible to demonstrate in human subjects an increase in antibodies to measles virus following the inoculation of the distemper agent.

Contemporaneously with this work on antigenic crossing between measles and distemper viruses in the United States, Polding and his associates in East Africa discovered that canine distemper and rinderpest viruses were also related. Later Plowright, also in East Africa, revealed a relationship between the rinderpest and measles virus when he found that in children, following an attack of measles, antibodies reacting with rinderpest virus developed in a high proportion of cases.

From the practical standpoint these biologically interesting relationships have stimulated investigation of the possibility of using distemper virus as an immunizing agent against measles. Adams in 1959 reported that the attack rate in an institutional population previously given live distemper virus vaccine was somewhat lower than it was in unvaccinated control groups. Hoenkenga and his associates in a trial carried out in Central America observed a slight reduction in the incidence of measles

in subjects who had received attenuated distemper virus vaccine but concluded that the potency of the distemper virus vaccine was too low to be useful. The use of canine distemper virus as a prophylactic against measles, therefore, does not at the moment appear promising. Further investigation, however, will be necessary before its consideration can be finally disregarded.

VACCINATION AGAINST MEASLES

Attenuation of the Virus. The newer knowledge of the measles virus, as I previously remarked, has been presented essentially by way of background for the trials of the attenuated virus vaccine in man which I shall now consider. These trials were carried out by us in collaboration with a number of investigators, or by others working independently with vaccines prepared in this laboratory from a virus attenuated in its virulence for monkeys.

This virus represents the distant progeny of a strain isolated from the blood of a patient and designated the "Edmonston" strain. When first recovered in human renal cell cultures, it failed to multiply in chick embryos but produced all the signs of measles in monkeys that I have described. After 24 passages in human renal cells followed by 28 passages in human amnion cell cultures, Milovanović of Yugoslavia, who was then working in our laboratory, showed that the Edmonston strain had gained the capacity to grow in the developing hen's egg. After 6 serial passages in the egg, Katz and his associates found, as might be expected, that this agent could be easily propagated in cultures of trypsinized chick embryo cells. During the first four passages in this system the virus exhibited no recognizable cytopathogenic properties, but subsequently caused cell-rounding, formation of small giant cells, and a peculiar morphological transfiguration that we called "spindle cell" formation. This latest effect had been noted early in human amnion cell passages and consists of an elongation of the typical epithelial cell into a shape reminiscent of the fibroblast.

When this chick cell-adapted virus was inoculated by the combined nasal and intravenous routes into susceptible monkeys no overt signs of infection were noted with the exception of a slight leucopenia. In systematic attempts to recover virus from the throat and blood of the inoculated animals none was demonstrated except in one monkey in which virus was detected in a single blood specimen. Obviously the reactions of the monkey to this chick adapted virus were in marked contrast to those induced by the agent when first isolated in human kidney cells. In spite of this absence of signs of infection, all monkeys that received it de-

veloped, by the 21st day, complement fixing and neutralizing antibodies in concentrations comparable to animals given the virus in its original virulent form. When challenged with virulent virus all of the vaccinated animals proved resistant as indicated by failure to exhibit any clinical signs of infection or demonstrable virus in the blood. These results indicated clearly that at some time during its prolonged sojourn *in vitro,* its virulence for the monkey had markedly decreased while the antigenic capacity was fully retained.

A pronounced difference between the capacity of the chick-adapted variant and its progenitor to persist and multiply in the central nervous system of the monkey was also demonstrated. In comparative experiments monkeys were inoculated with one or the other of these viruses into the hypothalamic region and into the cisterna magna. Thereafter specimens of spinal fluid, blood and throat swabbings were collected at frequent intervals and examined for the presence of the respective agent. Tests for antibodies were made at appropriate times. In the animals that received chick cell-adapted virus the agent failed to appear in the spinal fluid, nor was it demonstrated at any time in throat swabbings or blood. In contrast the parent virus was repeatedly recovered from the spinal fluid as well as other materials examined. It is particularly to be noted that antibody responses were comparable in both animals. Histological examination of sections of cord and brain taken 21 days after inoculation revealed no lesions attributable to either the virulent or attenuated agent.

First Trial of Vaccine. Because of its loss of pathogenicity for monkeys with retention of immunogenic properties we decided to explore the possibility of using in man the attenuated virus as a vaccine. We were reassured by these findings indicating that the capacity of the attenuated agent to multiply in the central nervous system of the monkey was clearly less than that of the virulent parent strain, since it would be *a priori* less likely to cause encephalitis in human beings.

Accordingly, we proceeded to prepare with the attenuated agent a vaccine to be used in an initial small trial in susceptible children. Attenuated virus in the 14th chick cell passage was grown in chick cell cultures nourished with the protein-free medium #199 of Morgan and Parker. To infected fluid taken from cultures during the period of maximal virus production a small amount of purified human serum albumin was added to stabilize viral infectivity. This material represented the vaccine which was preserved until use in the frozen state. After performance of the usual tests for bacterial contamination and animal toxicity, my associate, Dr. Samuel L. Katz, in October of 1958 administered the vaccine, with the

consent of parents or guardians, to 11 mentally defective institutionalized children who were regarded as susceptible to measles. Criteria for susceptibility consisted of negative history for measles and negative tests for neutralizing and complement fixing antibodies. Approximately 60 tissue culture infecting doses of virus were injected into each child by the subcutaneous route. As controls two susceptible children, who continued to remain intimately associated with the vaccinated group, were at the same time given sterile tissue culture fluid.

The clinical and serological events that followed in the vaccinated children may be summarized. No immediate reactions either at the site of inoculation or of a generalized sort were noted. After six — nine days, a rise in rectal temperature was recorded in eight of the vaccinated children which lasted from 2 to 5 days with a mean duration of 3.5 days. Maximal temperature recordings ranged from 100.8 to 103.8 F. with a mean of 102.8 F. After defervescence a pink, macular, nonpruritic, discrete rash af varying extent appeared in nine children. In five of them it might easily have escaped superficial examination. Koplik spots were seen in one child with a questionable rash. The rash appeared usually about the 11th day and lasted from 1 to 3 days. In four children it was seen only about the face and neck, in four others in addition to these areas the trunk was involved. In general the picture and of a mild, much modified measles. The most striking feature was the absence of any sign of disability. Notable, too, was the absence of any signs of involvement of the respiratory tract. In every case the child went about his usual routine with no loss of appetite or impairment of excretory functions. Two of the vaccinated children and the two control children exhibited no clinical signs of infection. Specimens of blood and throat washings obtained from each of the thirteen children on the 5th, 9th, and 15th day after vaccination were tested in tissue cultures for the presence of measles virus. None was demonstrated.

By testing specimens of serum taken from each child at appropriate intervals, emergence and decline of complement fixing and neutralizing antibodies were followed. Antibodies developed in all of the vaccinated children. Complement fixing antibodies were first detected on the 15th post vaccination day and attained the highest concentration recorded on the 22nd day. At the maximum, serum dilution titers ranged from 32 — 256. This range, as I shall again emphasize, approaches that exhibited by most individuals convalescent from natural measles. On the 36th day titers of neutralizing antibodies ranged from 90 — 300, again a range comparable to that most often encountered in early convalescence. At intervals

during the 2½ years elapsing since vaccination antibody levels have been determined. A curve of the geometric mean titers at these intervals is shown in Figure 1. It will be seen that from the maximum neutralizing antibodies declined by about a factor of 4 by the end of one year. Since that time, however, the level has remained essentially unchanged. The initial decline in complement fixing antibody was more pronounced and the titers finally attained were lower.

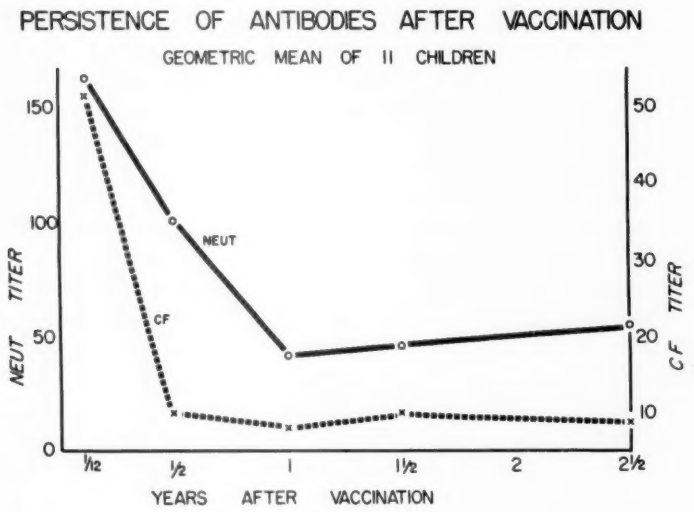

FIG. 1.

During this observation period of 2½ years, cases of natural measles have not occurred in the institution. We may confidently assume, therefore, that the vaccinated children have not been exposed. This assumption is also supported by the fact that tests for measles antibodies in the sera of the two control children have been consistently negative. Accordingly, the appearance and persistence of antibodies may be attributed to the effect of the vaccine.

From the results of this first small trial it was also concluded that:

i) The virus employed as vaccine is to be identified as the agent of measles.

ii) Under the conditions to which it was subjected in the laboratory, its virulence for man as well as for the monkey was reduced, but to a less degree.

iii) The virus is not transmitted from vaccinated subjects to susceptible contacts.

Additional Studies of the Original Vaccine. Because of the elevated temperatures noted in a few of the children in this first group an attempt was made to induce further attenuation of the virus. Accordingly, the vaccine strain was subjected to six additional chick embryo and four chick cell passages. A vaccine was prepared with virus from the last passage and, to distinguish it from the original vaccine or Vaccine A, it was referred to as Vaccine B. When Vaccine B was tested in susceptible children by Lepow and her associates in Cleveland, Ohio, they obtained the same spectrum of reactions which we had observed with Vaccine A.

Approximately 470 children and adolescents have now received parenterally either vaccine A or B. Since no difference in the effects of these vaccines was noted, we have assumed that all these subjects received comparable inocula. This large group of 470 children was made up of a number of small groups consisting of patients in institutions for the mentally retarded, patients attending a neurological clinic, patients with cystic fibrosis of the pancreas, and normal children in institutional schools and in the home. A number of investigators collaborated in these studies and have published or will soon publish their observations. We are greatly indebted to them for their interest and skillful management of these trials.

The relevant serological and clinical findings in the first 272 children in the series may be summarized as follows. To be first noted is the fact that many of the children did not respond to vaccination by the development of antibodies. Failure to respond was correlated in 101 of 107 cases with the presence of measles antibody in the pre-vaccination serum specimen suggesting that most of these non-reactors had previously experienced an infection. Of the 171 children in whose pre-vaccination sera no antibody was demonstrated, 165 or 96.5 per cent developed antibodies in significant concentrations following vaccination. We cannot clearly account for the failure of six initially negative reactors to respond. Possibly the quantity of virus in the inoculum was insufficient, but, more probably, pre-existing undetected low levels of antibody were responsible.

However this may be, it was evident that the incidence of serological response to vaccination among the susceptibles was gratifyingly high. As shown in the examples listed in Table 1, antibody concentrations attained after vaccination, approached those characteristic of convalescent phase sera from measles patients. The differences observed between the

mean antibody titers of the various groups probably depend in large part on technical factors.

The principal clinical reactions to vaccination among the children with initial negative serologic tests consisted of fever and exanthem. Fever usually began on the 7th or 8th day and lasted on the average about 3 days. The average maximal temperature by rectum was 102.4°F. The upper limit of 106°F. occurred in a single child who exhibited no other signs of illness and whose temperature quickly dropped after aspirin

TABLE 1. COMPARISON GEOMETRIC MEAN TITERS OF ANTIBODY AFTER NATURAL MEASLES AND MEASLES VACCINATION

Author	*Subjects*	*Stimulus*	*No. sera*	*Geo. M. Tit.**	*Range Tit.**
Bech[8]	Normal	Natural Measles	72	195	32-1024
Haggerty[9]	Normal	Vaccine A	32	121	16-256
Katz and Enders[5]	Mentally Retarded	Vaccine A	11	70	32-256
Black[3]	Mentally Retarded	Vaccine A	9	75	16-256
Krugman[6]	Mentally Retarded	Vaccine B	17	206	64-1024

* Reciprocal serum dilution end-point.

was given. In twenty-three or 14% of 165 children who developed antibodies no significant febrile response was recorded.

Only about one-half of the subjects developed a rash — definitely fewer than in our initial small trial. Again I would stress the fact that the rash usually appeared on the 10th to the 11th day when the temperature in most cases had returned to normal. Thus the interval between inoculation and appearance of rash corresponded with that noted by Francis Home. In appearance and distribution the rash was reminiscent of that associated with measles modified by gamma globulin. Koplik spots were seen only in a small proportion of the children. With very few exceptions the absence of disability was remarkable.

The results in two other groups who have received vaccine of the same manufacture deserve special comment. One consisted of children with cystic fibrosis who attended the clinic of Dr. Harry Schwachman at the Children's Hospital Medical Center, Boston, Massachusetts; the other of children suffering from a variety of neurological and metabolic disorders who attended the clinic of Dr. Frederic Gibbs and Dr. Ira Rosenthal of the Department of Pediatrics, University of Illinois College of Medicine, Chicago, Illinois.

The children with cystic fibrosis were chosen for vaccination because in such patients natural measles is often followed by serious disease of the lower respiratory tract which is frequently the seat of prior infection by bacteria or fungi. In Dr. Shwachman's clinic, as elsewhere in the United States, it is the practice in these patients to administer gamma globulin as a prophylactic measure after each exposure or presumptive exposure to measles, a troublesome and not always reliable procedure.

TABLE 2. CLINICAL REACTIONS AFTER MEASLES VACCINATION IN 125 PATIENTS WITH CYSTIC FIBROSIS

	No. of patients and incidence			
	Uncorrected		*Corrected*	
Reaction	*No.*	*%*	*No.*	*%*
None	51	41	26	26
Fever	37	30	37	37
Rash	5	4	5	5
Rash—Fever	32	26	32	32

An effective means of inducing active immunity, therefore, would be especially desirable under these conditions.

Measles vaccine has now been given to 125 patients with cystic fibrosis. Incidence of reactions to vaccination, as summarized in Table 2, was apparently somewhat less in this group as compared with that in the other children we have discussed. The difference, however, is probably to be attributed largely to the inclusion in the cystic fibrosis group of a number of initially immune children. Thus antibody determinations carried out on the pre-vaccination sera of 61 of these children indicated that about 20% were initially immune. Using this factor to correct the data for the entire group it is evident, as shown in the last column of Table 2, that the incidence of rash and fever approached that previously observed. Post-vaccination antibody titers in a sampling of 48 of the patients with cystic fibrosis who responded serologically to vaccination ranged from 1:8 to 1:512 (dilution of serum) or higher. These results suggest no impairment of the antibody-forming mechanism in patients with this disease. Unusually severe or abnormal reactions were not observed nor was the underlying illness affected in any way. It would seem, then, that individuals with this serious and debilitating illness tolerate the vaccine well.

A group of children were studied by Dr. Gibbs and Dr. Rosenthal with a special purpose in view: namely, to determine whether after

vaccination abnormalities in the electroencephalogram might occur. Previously Gibbs and his co-workers had found in a series of over 600 cases of natural measles accompanied by no clinical evidence of encephalitis that 51 per cent exhibited abnormal slowing of the brain waves. It was shown that these abnormal patterns were not due to fever *per se*. Gibbs and Rosenthal have now completed electroencephalograms in 40 children taken just before and 10 days after measles vaccination. Twenty-eight of the subjects were considered to be susceptible on the basis of serological tests. The reactions to the vaccine were entirely comparable to those I have already described. With a single exception no deviations from the prevaccination electroencephalographic patterns were noted. The exceptional child was suffering from a febrile upper respiratory tract infection at the time the second electroencephalogram was made. It has been stated by Gibbs that such infections may be accompanied by the type of transitory slowing that was recorded in this case.

These essentially negative electroencephalographic findings following vaccination led Gibbs and Rosenthal to conclude that fear of abnormalities in the electroencephalogram similar to those induced by natural measles can be excluded as a possible contra-indication to large scale use of the vaccine.

Prophylactic Effect of Vaccination. We may now ask how effective this vaccine has proved to be in the prevention of illness following exposure to measles. So far as we are aware no case of the disease has as yet developed in any of the approximately 500 children that have been vaccinated. Instances of known exposure, however, have not been numerous. Even so, there is available sufficient evidence to show that protection conferred by vaccination is of a high order. Thus, Krugman working in an institution for mental illness reported that none of 23 vaccinated children developed measles after intimate and prolonged exposure. In the same ward, 17 among 23 unvaccinated children came down with the disease and, in an adjoining ward, 29 among 50 unvaccinated inmates acquired measles. In Krugman's study the vaccine was administered about seven weeks before exposure occurred. Of the 23 vaccinated children who subsequently proved resistant on exposure, 18 children had exhibited the usual clinical and serological responses to vaccination.

Complete protection has also been observed by Lepow, Haggerty, and Schwachman among 35 vaccinated children considered to be susceptible at the time of vaccination. These children were exposed in the home to siblings with measles. The interval between vaccination and exposure in these family studies varied from 2 months to 16 months.

That the measles attack rate ranges from 75 to 95 per cent following exposure is known to everyone. Accordingly, these observations while limited seem sufficient to justify the conclusion that the attenuated virus vaccine prepared and administered in the manner I have described is a highly efficient prophylactic during relatively short periods. Only with the passage of time can the duration of protection be finally assessed. Nevertheless, the persistence of neutralizing antibodies for at last 2½ years as demonstrated in our first trial encourages us to believe that immunity may be durable, possibly as durable as that established by the natural disease.

Trials with Other Vaccines. Recently a number of other workers have carried out trials with living measles virus vaccines. Some of these have appeared in the literature; others are still in progress. Certain of the vaccines employed were prepared with our strain of chick-adapted virus but subjected to different conditions of cultivation. Others such as those tested by Smorodintsov in Leningrad consisted of entirely different strains. It is not my purpose to review these findings in detail. In general it can be said that with chick-adapted virus the characteristics of the clinical response have been broadly comparable to those I have described. Certain differences, however, were noted. For example, Schwarz and his co-workers isolated virus from the blood of one of two vaccinated children and Smorodintsev described frequent viral recovery from blood and throat washings from his subjects. McCrumb in Baltimore who studied a vaccine consisting of Edmonston chick-adapted virus that had been propagated for a few passages in canine renal cells, also encountered the agent more often in blood and throat washings. Hoenkenga and his associates employing chick cell vaccine reported that four poorly nourished children were sufficiently ill to be cared for in hospital during short periods. Satisfactory antibody responses were obtained by all these workers and several also reported a high level of resistance as measured by exposure to measles.

FUTURE DEVELOPMENTS

In this paper, I have attempted to outline some of the salient features of the history and present status of vaccination against measles. In conclusion we may consider very briefly what the future of vaccination may be. First it seems clear that the need for a simple, safe, and effective method for promoting lasting immunity will ensure continuation of efforts to perfect the vaccines now at hand. The need is emphasized merely by citing the incidence of measles in a country like the United States where

the annual reported attack rate outdistances that of any other notifiable disease. For example, about 5½ million cases were notified during the 10 year period from 1950 to 1959. The total incidence during this time is conservatively estimated at about 22 million cases. One has only to visualize the vast amount of discomfort hidden behind these figures as well as the additional burdens placed upon parents and the loss of time for all concerned, to be convinced that elimination of measles would be desirable.

When we add to such considerations the danger of serious and often fatal complications such as encephalitis, the need for a good prophylactic receives additional emphasis. To support this statement I may note that in the United States reported deaths from measles during the 3 year period from 1956 to 1959 totaled 1471, whereas deaths from poliomyelitis were recorded as 1042. Measles exceeded poliomyelitis mortality by about ⅓ during this period. Because measles encephalitis is known to vary with time and place, it is difficult to estimate accurately the incidence of this most serious complication. However, on what appears to be a fair assumption, encephalitis develops in about one out of 800 patients. On this basis I have calculated that about 28,000 cases occurred in the United States during the ten year period from 1950-1959.

In countries where nutrition and general sanitation are defective the frequency of pneumonia and other complications may be much higher with a corresponding increase in mortality. Under these conditions vaccination would seem to offer the only means of control that will be available for some time to come.

Because of the occasional high fever following vaccination, the discovery of means whereby such reactions may be eliminated will be a major objective of future research. Already trials are in progress in which modifying doses of gamma globulin are administered simultaneously with chick attenuated virus vaccine. Initiated by McCrumb, these trials are being extended by Krugman, Stokes, and Hilleman as well as others in the United States. It is even now apparent that with this procedure post-vaccinal reactions are considerably reduced and it has been shown that antibody formation is not usually suppressed although it may be somewhat diminished. Little or nothing is yet known regarding the degree or duration of the resistance that may be established by this technique. If found effective, sero-vaccination of this sort might be adopted in certain countries; but in others, it may be regarded as too complicated and too expensive. Much effort will be exerted, I feel sure, to push further the attenuation of the virus itself.

Finally, trials of the presently available chick cell adapted vaccine should be extended until they include sufficient numbers to give complete assurance that it is never encephalitogenic. Once its innocuity in this respect is demonstrated and its reactivity somewhat reduced we may predict that Home's ancient dream will at last become a reality.

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ALFRED S. EVANS*

The Wisconsin State Laboratory of Hygiene and the Department of Preventive Medicine, University of Wisconsin School of Medicine

INFECTIOUS MONONUCLEOSIS. OBSERVATIONS FROM A PUBLIC HEALTH LABORATORY†

FOREWORD

This paper is written in honor of Dr. John R. Paul for three reasons. First, in recognition of his contributions as a philosopher and educator in preventive medicine, and especially, the emphasis he placed on epidemiology as the basic discipline in prevention. To this end he developed and popularized the concepts of clinical epidemiology and serological epidemiology. Second, in tribute to the role he played both as a participant in and as a catalyst of research studies on infectious disease, and in particular, his investigations of infectious mononucleosis for which he discovered the only really diagnostic test—the heterophile antibody test—which bears his name and that of his associate Dr. Bunnell (the Paul-Bunnell test). Third, in appreciation of the role he played in my own career and development: fresh from my Army service in Japan, he gave me my first opportunity for research, stimulated and encouraged me in the four years I spent in his department at Yale, and provided guidance and assistance in the ten years after that. I am, therefore, honored to be afforded this chance to prepare, in his honor, this small contribution on infectious mononucleosis to which he first introduced me and to which, for better or for worse, I have been wedded ever since.

INTRODUCTION

The public health laboratory offers an excellent base for epidemiological studies of many types, particularly if the laboratory serves a wide geographic and population segment as does the Wisconsin State Laboratory. The large number of specimens which we receive for testing in bacteriology, virology, clinical chemistry, and cervical cytology laboratories provides insight into the epidemiology of both acute and chronic diseases. From information on

*Professor and chairman of the Department of Preventive Medicine; Director, Wisconsin State Laboratory of Hygiene. Formerly Research Assistant, Instructor, then Assistant Professor, Section of Preventive Medicine, Yale University School of Medicine, 1946-1950.

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our request slips, it is possible to learn the age, sex, geographic distribution, and often some of the clinical aspects of disease in about one half million persons each year. The major source of our specimens is from physicians' offices throughout the state so that the index of illness is based largely on non-hospitalized patients. The usefulness of the public health laboratory as a base for this type of epidemiological study will be explored in the present paper using infectious mononucleosis as the example.

EPIDEMIOLOGIC HYPOTHESIS

A concept of the epidemiology and pathogenesis of infectious mononucleosis has been recently presented[1]. In this hypothesis, the typical glandular form with its characteristic blood picture and rise in heterophile antibody represents the hallmark of fully developed illness corresponding to paralysis in poliomyelitis or to jaundice in viral hepatitis. There are three successive development phases (Fig. 1). Phase 1 is the characteristic, and often the only expression of clinical illness seen in childhood; it may be abortive. No further symptoms may appear, making diagnosis of infectious mononucleosis difficult, if not impossible. Clinically, fever, mild sore throat, and cervical lymphadenopathy may be present. The incubation period is relatively short (7-14 days). There is a high incidence of infectiousness in a group of susceptible individuals, and epidemics may occur which may go unrecognized without "Sentinel" cases in adults. The symptoms of this phase may be attributed to invasion by the virus and multiplication at local sites. Phase 2 is an intermediate one reflecting dissemination of the causative agent via the blood stream. Events in this viremic period may include a rash; implantation of the causative agent in secondary sites of multiplication such as lymph nodes, liver, and spleen; early lymphocytosis or perhaps leucopenia. The heterophile antibody is not yet detectable at this time, or is of low titer. The second phase may last about a week. The third and characteristic phase is then ushered in with fever, chills, and generalized adenopathy. With it there is marked lymphocytosis with abnormal forms, and if severe enough, a rise in heterophile antibody titer and abnormalities in liver function tests. This stage of illness is characteristic of the young adult who has escaped exposure earlier in life; it is the clinical type with which most physicians are familiar, particularly in student health services. In this age group the earlier phases may be absent or may be so mild as to go unrecognized, resulting in a long *apparent* incubation period of about 30 days before the onset of precise symptomology. The contagiousness of this form is low because the contacts of the same age are immune as a result of childhood

experience. Theoretically, the mild and inapparent primary infections should greatly outnumber the clinically characteristic type. This is certainly true of poliomyelitis and appears to be true of infectious hepatitis with which

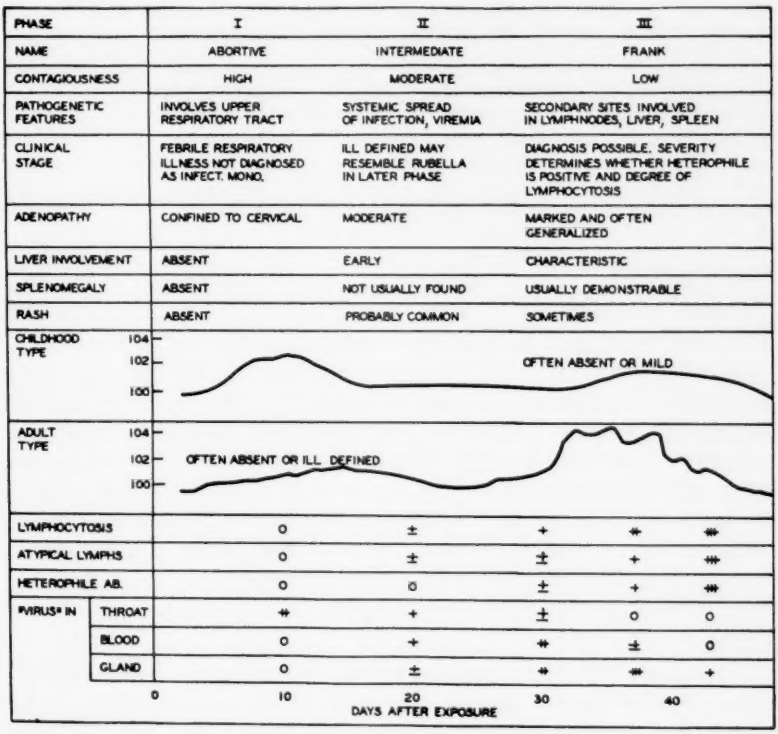


FIG. 1. Hypothetical Schema of the pathogenesis of infectious mononucleosis. (Reprinted by permission from *Amer. J. Hyg.*[1]).

infectious mononucleosis shares so many clinical features. The data obtained from a public health laboratory will be examined with this hypothesis in mind.

METHODS AND CRITERIA

Case. A test submitted for examination will be taken as equivalent to a case since duplicate tests have been eliminated.

Heterophile positive. A heterophile titer of 1 : 16 or above after guinea pig absorption is taken in this paper as the sole criterion for an established case. The clinical and

hematological data were not available to us, but since the physician submitted blood for this specific test, the assumption is made, in some parts of this paper, that the patient had clinical and/or hematological features suggesting infectious mononucleosis.

Heterophile negative or suspected case. This includes all patients without an elevation of the heterophile antibody. Again, for the purposes of discussion such cases will, in some places, be assumed to be suspected cases because a blood sample was submitted.

Population. All specimens for heterophile antibody testing received from Wisconsin residents from May 1960 through April 1961 were included, except those from the University Hospitals and the Student Infirmary. Epidemiologic aspects of infectious mononucleosis in the student group have recently been reported.[1] Because of the source of the specimens reported on in this paper, any conclusions drawn are subject to the limitation that the samples may not be representative of the cases which have occurred among the residents of Wisconsin.

OBSERVATIONS

During a one year period, from May 1960 to May 1961, sera from 387 individuals were positive for infectious mononucleosis with a heterophile titier of 1:16 or higher after guinea pig absorption. On the basis of a state population of 4 million, the *minimum* incidence rate in Wisconsin for serologically positive infectious mononucleosis is 9.7 per 100,000 per year. Many more positives have undoubtedly been found in hospital laboratories and are unknown to us. The figure of 9.7, however, is higher than the range of 1.6 to 5.9 per 100,000 found in England and Wales on the basis of 1,769 serologically proved cases from their Public Health Laboratory Service.[2] In University of Wisconsin students there is an admission rate for serologically proved cases of infectious mononucleosis of 252 per 100,000.[1]

A rough estimate of the extent to which the rate of 9.7 represents the actual incidence in the state can be obtained by calculations based on a known segment. Applying the rate in University students to the 553,400 residents of Wisconsin aged 15-24, as estimated from census data, one would expect 1,393 cases. Deducting the 50 University cases, which are excluded from our study, gives 1,343. This would be the *expected* number. The *observed* number of serologically positive cases in this age bracket, in this study, was 184. This suggests that the actual rate may be 7 times higher (1,343/184) than the observed, although the experience with the students may not be typical of the situation in Wisconsin generally. If the factor of 7 is appropriate the actual rate for all age groups would be about 68 per 100,000. This is close to the rate obtained from a careful survey of the general population of Oxford, England where the incidence was 56 per 100,000.[3] As shown in the discussion of geographic distribution

in the next section of this paper, the incidence in certain communities and counties of Wisconsin exceeded this.

In epidemics of infectious mononucleosis reported in the literature, the attack rate among those exposed has often been very high. For example, it was 80 per cent of 30 students in a boys school in 1923,[4] 60 per cent in 500 New Jersey students reported in 1925,[5] 89 per cent in an epidemic in an English Emergency Military Service hospital in 1943,[6] and 51 per cent (all subclinical) in 102 medical students in New York City in 1949.[7]

The 387 cases included in our estimate of incidence were all serologically proved and represented 11.1 per cent of the 3,480 specimens received for heterophile antibody testing. We have no information on whether the heterophile negative cases can be regarded as infectious mononucleosis or not because we have no clinical or hematological data on them. They were probably "clinically suspected" cases since the physician considered the diagnosis seriously enough to draw a blood specimen and send it to the laboratory for heterophile antibody determination. Assuming this to be true, then only 11 per cent of clinicallly suspect cases were heterophile positive. While this figure seems to be very low, it must first be remembered that these were largely milder cases seen in the physician's office or in the patient's home. Secondly, many younger age groups were included. These two aspects are in contrast to many studies dealing with hospitalized patients, or those restricted to a given age group such as university students or soldiers.

Information bearing on this point and on the ratio between clinical and subclinical infections is summarized in Table 1 from selected references. In sporadic and hospitalized cases the heterophile antibody has been present in about 50 per cent of the clinically diagnosed cases; in epidemics the frequency has been as low as 15 per cent[8] or even 3.8 per cent.[9] One outbreak of 27 presumed infectious mononucleosis cases is recorded without a single heterophile higher than 1:16.[10] There have also been outbreaks in which 53 and 68 per cent were heterophile positive.[6,7] Similarly, great variation in the subclinical to clinical ratio has been observed: it has varied from 13.8:1 to 1.6:1 in various epidemics and in one outbreak only *subclinical* cases were observed.[7] A ratio of 2 or 3 subclinical cases to one clinical case is a conservative estimate in epidemic situations.

GEOGRAPHIC DISTRIBUTION

In the year covered by this study specimens from residents of 190 Wisconsin communities scattered throughout the state were sent in for heterophile antibody testing. Of these, sera from residents of 72 different

TABLE 1. FREQUENCY OF HETEROPHILE POSITIVE CASES AND OF SUBCLINICAL CASES IN DIFFERENT STUDIES

Author	*Reference*	*Year*	*No. of cases*	*Epidemic or sporadic*	*Criteria for positive heterophile**	*Per cent positive** (Heterophile pos. blood clinical positive)†	*Ratio subclinical to clinical*	*Comment*
Stevenson & Brown	8	1943	123	E	1:8 Ab.	15.0	2.6:1	
Halcrow, *et al*	6	1943	296	E	1:64 Un.	68.2	1.6:1	Hospital outbreak, 38% over 1:64.
Vander Meer *et al.*	25	1945	356	E	>1:56 Un.	8.7	13.8:1	Military outbreak.
Gardner & Paul	12	1947	86	S	1:64 Un.	60.0	0:86	All clinical cases in hospital.
Watson, *et al.*	7	1951	51	E	1:28 Ab.	52.9	51:0	All subclinical cases medical students.
Goldthwait, *et al.*	9	1951	26	E	UNK.	3.8	4.2:1	Isolated soldiers. 1 case HA pos. at 1:1280. No subclinical case had positive hematological picture.
Niederman	13	1958	166	S	1:80 Un.	43.4		All clinical cases in hospital.
Hobson, *et al.*	3	1958	398	S	1:80 Ab.	60.8		2-year community survey in England. 100 cases HA neg., blood pos.; 56 clinical pos. only, 43 asymptomatic.
Evans	1	1960	305	S	1:16 Ab.	56.0		Hospitalized students. 27% HA neg., blood pos. 17% clinical pos. only.

* Heterophile antibody (HA): positive at this titer or higher. Ab. = titer after guinea pig kidney absorption. Un. = unabsorbed titer. UNK. = unknown. In some papers the exact level was not entirely clear.

** The denominator for this percentage figure is the total number of clinical diagnoses as derived from the author's data.

† Fulfill criteria on a clinical, hematological, and perological basis.

communities were serologically positive for infectious mononucleosis. The incidence in individual communities varied widely as shown for eight areas in Table 2; the highest, in which at least 5 cases were positive, was in Oconomowoc with 10 cases or a rate of 149 per 100,000; in Madison it was 64.5 On a county basis the highest incidence was in Dane County, which includes the City of Madison, with 53.1 cases per 100,000 (90 tests positive out of 689 done). High rates per 100,000 in other counties included Columbia County with 44 (15 positive out of 157), Green County with

TABLE 2. THE INCIDENCE OF SEROLOGICALLY POSITIVE INFECTIOUS MONONUCLEOSIS IN SEVERAL WISCONSIN COMMUNITIES

	Number of tests				
Community	*Done*	*Positive*	*Per cent Positive*	*Population*	*Incidence per 100,000*
La Crosse	95	14	14.7	47,575	29.2
Madison	554	82	14.8	126,706	64.5
Marshfield	71	12	16.9	14,153	84.8
Milwaukee	273	34	12.5	741,324	3.4
Oconomowoc	20	10	50.0	6,682	150.0
Portage	30	4	13.3	7,822	50.6
River Falls	31	5	16.1	4,857	102.0
Sheboygan	18	10	55.6	45,747	21.9

33 (8 positive out of 22), and Ashland County with 30 cases (6 positive out of 27). Examination of the *monthly* incidence for these areas, as well as for the 71 other areas with positive cases, revealed no evidence suggestive of an outbreak. In cities like Milwaukee, the apparent rate was low as most specimens were sent to local laboratories for testing.

SEASONAL INCIDENCE

Figure 2 depicts the monthly distribution for heterophile positive sera and heterophile negative sera. March, April, May, and July were the months with the highest number of heterophile positive sera. In University of Wisconsin students, March, April, and May have also been months of high incidence[1] but an additional feature of this student group, as well as of Smith College students,[11] has been the sharp rise occurring a month after a vacation period—that is, in October after the summer vacation, and in February after the Christmas vacation. The trend has not been noted in the present study nor at the Grace-New Haven Hospital where cases have been studied by Paul and his colleagues.[12,18]

The manner in which the monthly distribution of the heterophile positive specimens paralleled the distribution of the heterophile negative specimens lends credence to the possibility that the latter actually represent *cases* of infectious mononucleosis (Figure 2). These suspected cases outnumber the proved cases by a factor of 15.7 :1 in January to 5 :1 in July. Subsequently this observation will be examined further.

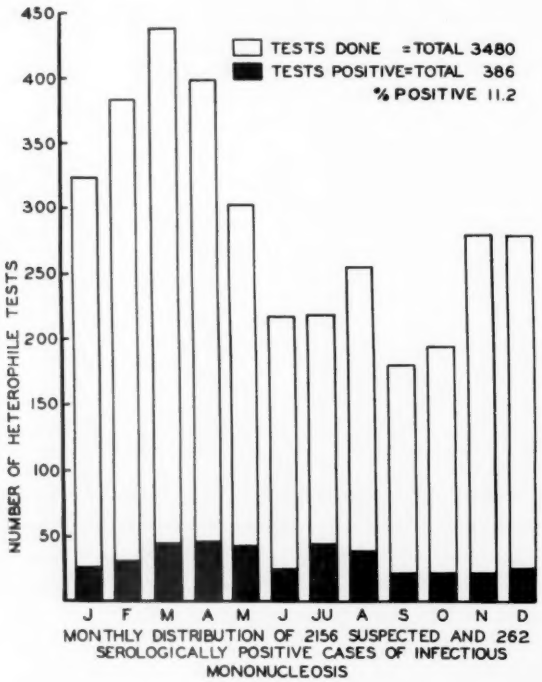


FIG. 2.

A review of the literature on epidemics of infectious mononucleosis points to a preponderance in January-February-March[14-17] but there is much variation. The tendency for both sporadic and epidemic cases to occur in late winter and early spring is in contrast to the seasonal pattern of infectious hepatitis and poliomyelitis. The former shows a sudden increase in prevalence in the late summer and autumn, often building up to epidemic proportion during early winter and soon declining; the latter occurs in both epidemic and non epidemic forms, most commonly in the summer, in

both northern and southern temperature climates; however, in tropical areas cases occur more or less uniformly throughout the year.

SIGNIFICANCE OF AGE

Age per se. The age distribution of serologically proved and "clinically suspected" cases* of infectious mononucleosis is presented in Figure 3. The

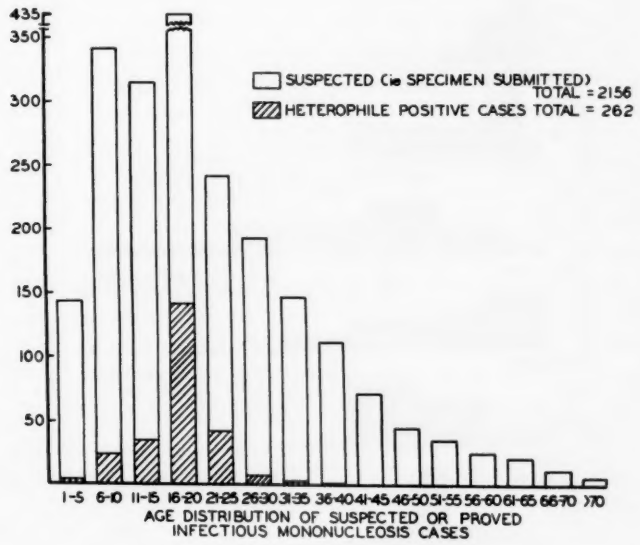


FIG. 3.

finding of a predominance of serologically proved cases in the young adult confirms many other reports. The youngest case in this study was a 3-year-old and the oldest case was 43. In Newell's report from the Public Health Laboratory Service in England, 61 per cent of 1,689 serologically positive cases were between 15 and 24 years, 18 were under 15 years.[8]

If one looks at the age distribution of the "clinically suspected" cases, infectious mononucleosis appears to be a *childhood* disease reaching a peak incidence in the late teens after which it declines steadily into middle and old age. In this context the heterophile response may be an age-conditioned host response of the young adult and may reflect a particular phase in the

* This term is used to indicate heterophile negative patients.

development of our lymphatic and/or immunological mechanisms. The frequency of heterophile negative cases in childhood fits the concept of pathogenesis earlier expounded.

Through the years paralytic poliomyelitis has shown a relative shift to a progressively older age group.[18] But has the age distribution of in-

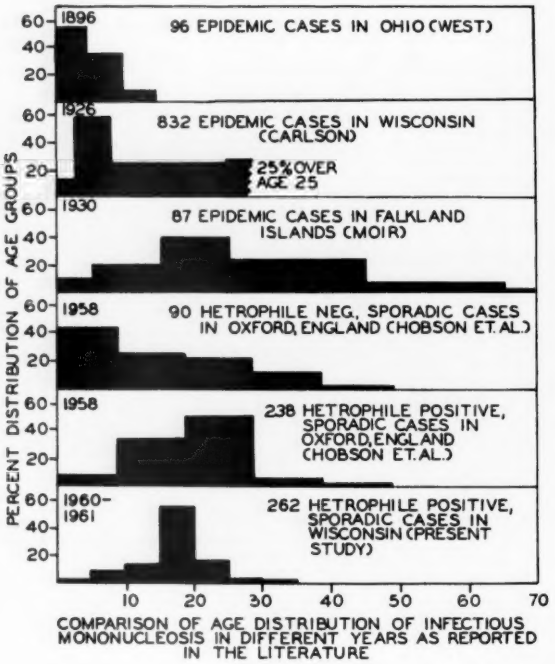

1896
96 EPIDEMIC CASES IN OHIO (WEST)
1926
832 EPIDEMIC CASES IN WISCONSIN (CARLSON)
25% OVER AGE 25
1930
87 EPIDEMIC CASES IN FALKLAND ISLANDS (MOIR)
1958
90 HETROPHILE NEG. SPORADIC CASES IN OXFORD, ENGLAND (HOBSON ET AL.)
1958
238 HETROPHILE POSITIVE, SPORADIC CASES IN OXFORD, ENGLAND (HOBSON ET AL.)
1960-1961
262 HETROPHILE POSITIVE, SPORADIC CASES IN WISCONSIN (PRESENT STUDY)
PERCENT DISTRIBUTION OF AGE GROUPS
60
40
20
10 20 30 40 50 60 70
COMPARISON OF AGE DISTRIBUTION OF INFECTIOUS MONONUCLEOSIS IN DIFFERENT YEARS AS REPORTED IN THE LITERATURE

FIG. 4.

fectious mononucleosis shown a similar trend? Data collected from epidemics or surveys of communities in which all age groups were presumably exposed is shown in Figure 4. In the early outbreaks, such as those described in Ohio in 1896,[15] in Wayne County, New York State in 1925,[19] and in Appleton, Wisconsin, in 1926,[17] the highest incidence occurred in children. Indeed, Tidy and Morley have calculated that at least 80 per cent of all cases reported up to 1921 were under 13 years of age.[20] The outbreak described by Moir in the Falkland Islands in 1930[21] is of special interest in that this was an isolated and presumably susceptible community. As shown

in Figure 4, the cases were distributed through all age groups and 10 per cent of the cases were over 45 years of age. This is similar to the wide age distribution of paralytic poliomyelitis in an isolated and susceptible group of Arctic Eskimos.[29] In more recent years no satisfactory description of an *epidemic* of infectious mononucleosis can be found in which all age groups were exposed. However, the age distribution of infectious mononucleosis as reported by the general practitioners of Oxford, England shows 2 patterns (Figure 4). In the heterophile negative patients, children were primarily involved with over 40 per cent of the cases under 9 years of age. In the heterophile positive cases, 50 per cent were in the 20-29 age group. This age difference is similar to that found in the present study between heterophile positive cases and the heterophile negative "clinically suspected" cases. This general age pattern is consistent with the hypothesis that infectious mononucleosis is a common disease of childhood which is infrequently recognized because it may be mild, abortive, and present with a negative heterophile; in the young adult a concentration of heterophile positivity as well as more severe cases are characteristic and the consequence of delay in exposure until later years. This is a well recognized pattern in certain other infectious diseases—e.g. poliomyelitis, hepatitis, varicella, mumps, etc., in which adults usually experience more severe manifestations than do children.

Age as Related to Other Factors. No relation between the age of the serologically positive case and the month of the year could be demonstrated. A comparison between age and the height of the heterophile antibody titer showed that high titers were uncommon in the young age group. Under age 5 no titer over 1:128 was observed, and between the ages of 6 and 10, only 7 were seen. The peak titers, as well as the most frequently positive titers, were seen in the 16 to 20 age bracket. Up to age 30, both the frequency and the height of the positive heterophile tests increased with age.

SEX DISTRIBUTION

Specimens from 1,681 males were tested of which 200 (11.9 per cent) were serologically positive after guinea pig absorption. Specimens were received from 1,796 females of which 185 (10.3 per cent) were serologically positive. The male: female ratio was 1.1:1. In some months, for example July, positive sera from females outnumbered positive from males by 2:1; in other months, such as January, the reverse was true. A sharp peak in positive sera from females occurred in July while the peaks of positive sera from males were wider spread in March, April, and May.

In his review of the literature, Bernstein indicates a 3:2 male predominance over females.[28] This ratio appears to be based on actual numbers of cases and not on a sex-specific rate. In Wisconsin students the M:F case ratio is 1.4:1, but the sex-specific rates per 1,000 students per year are 2.02 for males and 3.2 for females.[1]

FAMILIAL OCCURRENCE

Two instances of more than one case in the same family were identified in the present series. In one, a male of unknown age had an absorbed titer of 1:16 and this was followed 59 days later by illness in a 32 year old female who developed an absorbed titer of 1:1024. In the other family, a 6-year-old girl with a titer of 1:64 was found in December 1960 and 109 days later her 8-year-old brother developed a titer of 1:1024 which was still elevated to 1:512 on retesting a month later.

Historically, infectious mononucleosis has been common in families. This was true of Pfeiffer's first description in 1889 in which 4 cases were described in one family;[24] of the Ohio outbreak described by West in 1886 with 43 cases in 96 families;[25] of the 1924 outbreak in New York with 63 of about 100 cases in families;[19] and of the 1924 Wisconsin epidemic with 2-7 cases in each of 64 families.[17] In more recent years, the familial occurrence has not been so clear cut; very few community outbreaks of infectious mononucleosis have been reported and most others have been centered in groups isolated from the family circle—university students, military, etc.

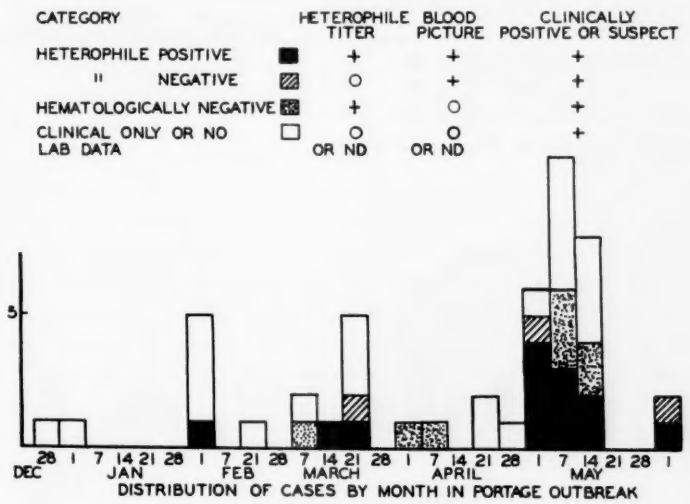
A WISCONSIN "EPIDEMIC"

In early 1961 there was trouble in Portage City. An outbreak of illness believed to be infectious mononucleosis had been reported. A similar story came from Wisconsin Dells. On 20 May 1961, Dr. Joseph Priezler, State Epidemiologist, and I investigated the situation. Physicians in one large clinic, and one private general practitioner were interviewed and their records examined; the local hospital laboratory was visited, and laboratory data reviewed.* Three patients were examined, but they were late in the course of illness and characteristic clinical findings were absent.

The clinic in Portage had seen 50 suspected cases of infectious mononucleosis from January to May, and the local hospital laboratory had tested the sera from many of these; in all, 49 in-patients and 24 out-patients were

*I wish to express my appreciation for the fine cooperation given by Dr. Josef Preizler, Dr. Joseph W. Pavelsek, Dr. Ray Rueckert, Dr. C. F. Broderick, Nurses Mrs. Susan Waisman and Miss Sonja Smith, Sister Concordia, and Mr. George Bixby.

tested. Records of absenteeism from high schools were obtained through the cooperation of the county nurse and a questionnaire was sent to students missing school since January which requested detailed clinical data about their illnesses. From all available information there were 52 cases clinically diagnosed as infectious mononucleosis.** On the basis of the population of Portage of 7,334, this represents a rate of 709 per 100,000 for the 6-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DEC JAN FEB MARCH APRIL MAY
DISTRIBUTION OF CASES BY MONTH IN PORTAGE OUTBREAK

FIG. 5.

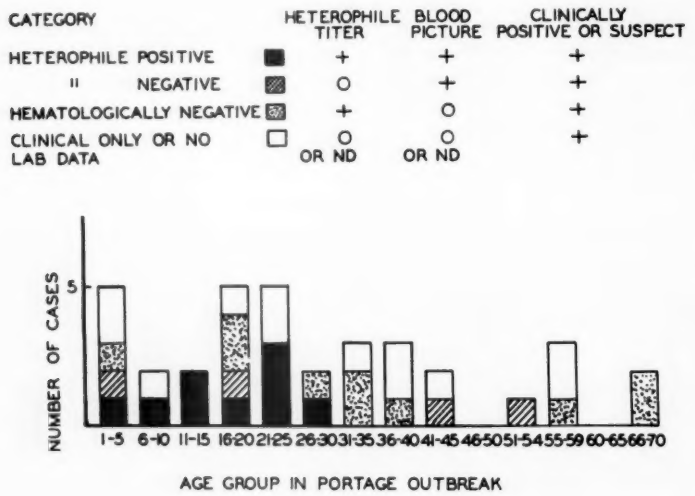
month-period of the apparent outbreak. In 13 of the cases, an elevated heterophile titer and a positive hematological picture were found in local laboratories.† In 4 others, only hematological evidence was found, and in 9, only elevated heterophile titers supported the clinical diagnosis. In the remaining 26, the diagnosis was made on clinical grounds entirely because laboratory studies either were not done, or did not meet the criteria outlined. The monthly distribution of cases is given in Figure 5. Three peaks about a month apart are seen with a major peak of 25 cases in May. In Figure 6 the age distribution reveals a wide spread but with two-thirds of

**A case was considered clinically positive if fever, sore throat, and cervical adenopathy were present.

†A heterophile titer of 1:160 was taken as elevated using unheated serum and formalin treated red cells from a commercial source in a plate dilution technique. A hematological picture was considered positive if the percentage of lymphocytes and monocytes was 50 or more.

the serologically positive cases in the 11-25 age bracket and none over age 30.

The main clinic serving Wisconsin Dells had seen 126 clinically suspected cases since January, 10 of whom were hospitalized. Accepted at face value, this would be a rate of 6,438 per 100,000. The cases were concentrated in March and April. Serological evidence confirmed the diagnosis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NUMBER OF CASES
5
1-5 6-10 11-15 16-20 21-25 26-30 31-35 36-40 41-45 46-50 51-54 55-59 60-65 66-70
AGE GROUP IN PORTAGE OUTBREAK

FIG. 6.

in 7 with a titer of 1:112 or more by the Davidson technique and the 2 blood counts done on these patients showed over 50 per cent lymphocytes. In 10 others the hematological picture was diagnostic but the heterophile titer was not elevated over 1:56. Two of these occurred in a family, and tests on 4 other family members showed suggestive fiindings.

There were three disturbing aspects to the outbreaks in both Portage and Wisconsin Dells: i) it was too late to make a clinical appraisal of the patients through personal examination; ii) heterophile titers were often elevated in the face of a normal blood picture; iii) retesting in the State Laboratory of 15 serum samples found to be at least 1:80 in local laboratories were negative at 1:8 even before guinea pig absorption. Were these, in fact, outbreaks of infectious mononucleosis or not? Clearly there had been a number of persons with undeniable clinical and hematological features of this disease. In some, the elevated heterophile titer was probably valid;

in others false positive tests were obtained due to a technical error in the method employed. (Commercial formalin treated red cells and unheated sera were used.) There was also over enthusiasm in making a clinical diagnosis and a suggestion of public hysteria, and there was absenteeism in the high schools. It is quite possible that as word got around about the symptoms of "the mono", many a tired housewife was sure she had it, and many a high school student found it a good excuse to stay home. This outbreak is probably characteristic of community outbreaks of ill defined illness where the precise nature challenges the epidemiologist and public health officer. Without exact and specific immunological tests, both the basic nature and the extent of epidemics of diseases of unproved etiology will remain an enigma.

SUMMARY

Certain epidemiological features of infectious mononucleosis as seen in a public health laboratory are discussed in relation to available information in the literature. Among these are incidence, age, sex, and season. The concept is emphasized that infectious mononucleosis is a disease with a broad clinical spectrum in which the glandular, heterophile, and hematological features may, in fact, only represent an age conditioned host response of the young adult.

An outbreak of illness with clinical features resembling infectious mononucleosis is presented, and the opportunities in the public health laboratory for epidemiological studies is emphasized.

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SVEN GARD*

Department of Virus Research,
Karolinska Institutet Medical School,
Stockholm, Sweden

EXIT POLIOMYELITIS—WHAT NEXT?

POLIOMYELITIS IN SWEDEN

For the first time in 60 years we can afford to take an optimistic attitude towards the poliomyelitis problem and its solution. In Sweden, notification of poliomyelitis was introduced in 1905 and separate reporting of paralytic and non-paralytic cases has been practiced since 1936. The records are probably at least as complete and reliable as anywhere else in the world. A graphic representation of the annual numbers of reported cases is found in Figure 1.

In the period of 56 years covered by the diagram, the annual number of cases has fluctuated considerably but certain trends may be traced. Larger epidemics appear at roughly 5-year intervals, and particularly extensive outbreaks are followed by a few calm years, especially noticeable after the 1911-12 and the 1953 epidemics. Furthermore, the years after the 1911-12 epidemic were characterized by a gradual increase in attack rates and a gradual shift in age distribution of the paralytic cases. Counted on 5-year periods beginning in 1916 the average annual reports, in chronological order, amounted to 286, 347, 490, 703, 1293, 1714, 1424, and 1526 cases. In 1911 only 12% of patients with paralysis were over the age of 20 years; the corresponding figures were 31% in 1934; 48% in 1944; and 57% in 1953.

Vaccination Campaigns. Against this background, the course of events after initiation of mass immunization with inactivated vaccine in the early spring of 1957 assumes a definite significance. The approximate numbers of persons given the primary two inoculations were 717,000 in 1957; in consecutive subsequent years 358,000, 1.039.000, 620.000, and 1.200.000. Hence, as of June 3, 1961, a total of about 4 million out of a population of 7.5 million. The first program comprised mainly children born in 1949-53; the campaign was later gradually extended and now covers all persons born in 1910 or later who are entitled to vaccination free of charge. The approximate coverage as of June 1961 is, in the 6-month to 6-year-old

* Professor of Virology. During part of 1939, a Rockefeller Fellow in the Section of Preventive Medicine, Yale University School of Medicine.

group, 70%; in age groups from 7-19 years, about 95%; in the groups from 20-39 years, about 60% and from 40-51 years, about 45%.

Except for 1957 when about 650,000 children were given imported vaccine, only a domestic product has been used. In the former group a total of six cases of paralytic poliomyelitis have been reported, while so far not a single verified case has occurred among a total of about 3.3 million persons who have received at least two doses of Swedish vaccine. The total number of paralytic cases in the pre-vaccination period from 1936 to 1956

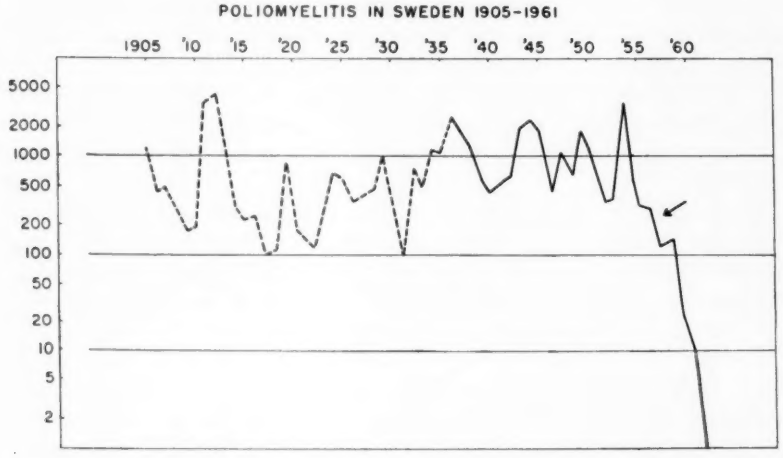

FIG. 1. Arrow indicates beginning of mass vaccination with inactivated vaccine.

varied betwen 300 and 3,400 annually with an average of 1136 cases; since vaccination was introduced, the figures reveal 124 in 1957, 156 in 1958, 25 in 1959, 10 in 1960, and 0 in the first half of 1961.

This dramatic drop in case incidence to less than 1% in four years was not expected. Even with a wild hope for a 100% protective effect—which actually seems to have materialized—one could not have anticipated a concommitant drastic reduction in attack rates among non-vaccinated persons. However, from the annual average, 15 per 100,000 before introduction of the vaccine, the case rate among non-vaccinated individuals dropped to 0.21 per 100,000 in 1960. It has to be taken into account that the age groups which previously had the highest attack rates are those most thoroughly covered by vaccination. As a result the age distribution of paralytic cases shows a further shift, with about two-thirds now in persons over 20 years. However, this fact alone cannot explain the phenomenon; even in the best

immunized age groups, the fraction of non-vaccinated individuals exceeds by four times the ratio of the 1960 attack rate to the previous average.

Virus Dissemination in the Community. Most likely, the reduction in attack rate means but one thing—a reduction in the dissemination of wild poliovirus strains in the country. A rational attempt to prove or disprove such a suspicion would probably be by way of statistically weighted sampling in repeated nation-wide surveys. Investigations of this kind have not been performed and the only data available at present are the re-

TABLE 1. RECOVERY OF ENTERIC VIRUSES FROM HOSPITALIZED PATIENTS IN 6-MONTH PERIODS, JANUARY 1958 THROUGH JUNE 1961. RESULTS FROM VIRAL DIAGNOSTIC LABORATORIES IN 5 AREAS OF SWEDEN

	1958		*1959*		*1960*		*1961*
	1st	*2nd*	*1st*	*2nd*	*1st*	*2nd*	*1st*
No. paralytic cases	156		25		10		(0)
No. specimens	—		5224		3962		2233
No. strains							
poliovirus	9	117	42	39	6	10	1
non-polio-enterovirus	5	80	14	22	3	38	28
adenovirus	8	31	45	235	50	52	16
nontyped nonpoliovirus	2	88	22	17	5	0	0
Poliovirus % recovery	(8.7)*		1.5		0.25		0.045

* Data from 1958 not quite comparable to those of subsequent years. Cf. text.

ports from the Public Health Laboratories in which virus diagnostic work is carried out. Five such laboratories operate in Sweden, each serving a different region.

This material does not meet even modest requirements of random sampling. First, it refers almost exclusively to hospitalized patients. Since suspicion of poliomyelitis with or without paralysis still is one of the primary reasons for requests for virologic examinations, a principle of selection is thereby introduced that presumably would favor the detection of any circulating strains of poliovirus. However, it is impossible to evaluate the amount of bias thus introduced or even to be certain about its direction. Actually, in some hospitals, stool samples are examined routinely in the virus diagnostic laboratory so that many samples tested did not come from suspected cases of poliomyelitis. Furthermore, the nature and case incidence of prevalent diseases will affect the extent of sampling as well as geo-

graphic and age distribution involved. Finally, remote regions will tend to be largely under-represented.

However, if in full awareness of the weaknesses of the material at hand, the reports of virus isolations from stools (and a small number of throat swabs) over the period January 1958 through June 1961 are compiled, the results are as shown in Table 1. The following facts should be pointed out.

1) The number of poliovirus isolates is roughly in the same range as the number of paralytic cases reported. In 1958 the occurrence of a case still did not regularly incite a search for virus in the patient's environment. In later years, as the number of cases dwindled, more interest was directed toward investigations of the dissemination of poliovirus among household and other contacts and a number of strains were thus recovered. It should be pointed out that the majority of strains isolated from other than paralytic or nonparalytic poliomyelitis patients were from healthy contacts of cases. Very few isolations have been reported from individuals with no connection with a known case, the only representatives of the latter category being 7 strains of type 3 virus recovered in 1959 by Kjellén (personal communication) in a systematic search for various viruses in a children's hospital clientele.

2) The number of unidentified, non-poliovirus, cytopathogenic agents has steadily decreased over the years, indicating improving diagnostic facilities. For various reasons it may be assumed that in Sweden most of these agents belonged in the adenovirus group.

3) The wave of adenovirus infections in 1959, almost exclusively type 7, is conspicuous.

4) The number of non-polio-enterovirus isolates has been very moderate. However, the relatively high recovery rate in the first 6 months of 1961 might indicate that these viruses now are on the increase.

5) Whereas the seasonal distribution of isolates of the other enteroviruses is uneven—with 6 to 7 times more positives in the second than in the first half of the year—a similar distribution of poliovirus isolates is apparent only in 1958 but not at all in 1959 and 1960.

As already pointed out great caution must be used in interpreting these observations. From the age distribution of serologic immunity found in surveys of pre-vaccination samples[1], the density of exposure during the pre-vaccination era can be estimated at an average annual infection rate of the order of about 10% to 20% of the susceptible population for each one of the three types of poliovirus.

These figures represent the accumulated rates over the whole of one year. Obviously the actual rate of infection at any one particular time would be lower. Provided the sampling were uniformly distributed over the seasons,

and the duration of virus excretion taken into consideration, the over all annual recovery rates should be expected to be of the order of about 5%.

The actual recovery rates in Table 1 were 8.7% in 1958, 1.5% in 1959, 0.25% in 1960, and 0.045% in the first half of 1961.

As already pointed out these figures cannot be taken at their face value. As the fraction of susceptible individuals in a population decreases with age, the infection rate must be expected to show a pronounced age dependence, a feature clearly illustrated by Table 2. Obviously the age distribution of the population sampled will strongly affect the over all

TABLE 2. POLIOVIRUS ISOLATIONS FROM STOOLS OF HOSPITALIZED PATIENTS IN RELATION TO AGE OF SUBJECT. (STATE BACTERIOLOGICAL LABORATORY, STOCKHOLM, 1958)

Age group	*No. specimens*	*Positive*	*% pos.*
0-6 years	110	27	24.5
7-19 "	197	16	8.1
20-49 "	313	21	6.7
50+ "	77	3	4.0
Not stated	97	2	
Total	794	69	8.7

recovery rates and, thus, the comparability of results. Examination of the material in Table 1 in this respect has shown the proportion of the pre-school age group to vary between 10% and 14%, and that of persons under the age of 20 between 39% and 46%. Apparently these variations are too slight to explain the 35-fold difference between the recovery rates in 1958 and 1960.

What is remarkable about Table 2 is not the correlation of percentages of poliovirus isolations with age of the subjects, which was to be expected, but rather the very high recovery rates themselves. In 1958 the paralytic attack rate in non-vaccinated persons was 2.4 per 100,000, that is, about 17% of the pre-vaccination average. More than 90% of the strains isolated were of type 1 and the great majority of them derived from paralytic cases. Under these conditions an over all recovery rate of 25% in the pre-school age group indicates that, except for poliomyelitis, few diseases calling for virologic examination were prevalent in 1958. Consequently, the mode of selection should introduce a strong bias favoring detection of polioviruses and the recovery rates could not be considered representative of the occurrence of poliovirus infections in the population at large. In 1959 the situation was different. The wave of type 7 adenovirus infections dominated

both in the hospital records and in the virus laboratory reports. Under these conditions there is no reason to suspect an over-representation of poliovirus isolations. On the other hand, it is not likely that adeno- and polioviruses interfere one with the other and the conclusion would be, that the figure in Table 1 might reasonably be a relatively true measure of the actual poliovirus dissemination. Apparently, in 1960 neither enterovirus nor adenovirus infection of the type now under consideration was prevalent; and in this instance too, we lack reason to suspect bias.

At the time of writing, there are no further detailed data for 1958 other than those entered into the table. Thus, the recovery rate listed within parenthesis does not refer to the total material but only to the laboratory providing the material for Table 2. The true figure is, most probably, of the same order, but this fact is a further reason for a cautious evaluation of the trends. Taken together, all this seems to justify only a vague assumption of a trend towards a gradual reduction in the dissemination of polioviruses in the period under study. Nonetheless, these data may be adduced as evidence supporting other independent observations.

As a matter of fact such independent although indirect evidence exists. In 1958 and 1959 pre-vaccination immunological surveys of groups with about 100 children in each, children 3 to 5 years old, were conducted in four different places in central Sweden. As far as antibodies to type 1 polioviruses were concerned, antibodies were detected in 0.1, and 3% of three groups studied. The fourth group came from a town where a small outbreak of type 1 paralytic poliomyelitis had been observed in the fall of 1958. In this group, examined in the following spring, 20% had antibodies to type 1. These findings indicate that during the preceding 3 to 5 years little or no virus had been circulating in the society except in connection with scattered local outbreaks of clinical disease.

Similar conclusions can be drawn from some previously reported results of field tests with live type 1 (CHAT) poliovirus vaccine: in a school for blind children 90% of the age groups from 7 to 11 years, and 60% of the 12 to 15-year-olds proved susceptible to infection[8].

To all appearances, therefore, the incidence of paralytic poliomyelitis can serve as a measure of the rate of dissemination of type 1 virus in the country, also in the post-epidemic period after 1953, which would mean a drastic reduction when compared to the preceding 20-year period. The immunological surveys indicate that the same might be true of type 2, whereas dissemination of type 3 probably has been somewhat less restricted. The only reported isolations without some association to known cases of poliomyelitis were the group of seven type 3 strains found in 1959 and mentioned above.

Theoretical Considerations. If these conclusions are accepted as probable, it remains to find an explanation for the phenomenon of steadily declining circulation of poliovirus. Apparently, as borne out by immunological surveys carried out in 1957 before initiation of the vaccination campaigns, the trend was then already established. This should not be surprising. The 1953 epidemic was more severe than any previous outbreak in Sweden, and unlike those occurring during the preceding 20-year period which generally were focal, it involved the whole country. Presumably, therefore, an extensive immunization of the population had taken place, and a considerable resistance against dissemination of the virus type which was responsible for the big epidemic should have been built up. In the following years other enteroviruses dominated the picture, especially in 1957 when 13,000 cases of aseptic meningitis were reported, cases with or without rash, associated mainly with ECHO virus type 9. It is thus conceivable that interference from other enteroviruses might have had a suppressive effect on the spread of polioviruses, including those types to which no specific resistance was present in large segments of the population.

In 1958, however, none of these restricting factors should have been much in evidence. By then a population of pre-school age children had accumulated, close to 100% susceptible to all three types of poliovirus. Other viruses, potentially capable of interference were scarce. Opportunities for introduction of virulent strains of virus clearly existed, as epidemics actually attacked Norway and Finland in 1958. As already mentioned, a few local outbreaks did indeed occur, and some cases were identified as apparently imported from Finland. However, in each such case the outbreak remained focal and no spread of virus seemed to occur beyond rather narrow geographical boundaries.

Under these conditions a discussion of the possible effects of mass immunization with inactivated virus can hardly be avoided. Admittedly, it is generally assumed that, while the use of live poliovirus vaccine can be expected to restrict radically the circulation of wild virus to the point of eradication, immunization with inactivated vaccine neither produces complete resistance to the natural infection nor does it affect significantly, the multiplication of virus in the intestinal mucosa or its excretion in the feces. Thus, inactivated vaccine would not be expected to interfere with the dissemination of wild virus strains. The question now raised is whether or not this assumption is correct.

At the First International Conference on Live Poliovirus Vaccines in 1959 the results of the early Swedish small scale trials with live CHAT type 1 virus were reported.[8] It was then pointed out that in persons without previous experience with live virus, but with antibodies after vaccination

with inactivated vaccine, the course of infection was to a certain extent dependent on the actual antibody titer at the time of exposure to live virus. While none of the persons in this category were resistant to infection, the average duration of the excretion following feeding of the virus was inversely related to the actual titer of circulating antibodies. As pointed out by Bodian, this observation is in accord with Howe's finding concerning the rate of type 2 virus excretion in artificially immunized chimpanzees[4].

In 1958 Salk[5] also speculated over the declining attack rates in non-vaccinated individuals which had been reported from several countries where large scale vaccination had been initiated. As it later turned out, after the "sobering experience" of the 1959 epidemic in the United States[6], the optimistic interpretation these earlier observations received in many quarters was hardly justified. In any event, Salk was inclined to regard the reduced attack rates as an effect of the vaccination, reasoning as follows. "If oropharyngeal secretions are more important for transmission of virus in communities where contact with sewage or feces is less likely, and if vaccination has little or no effect upon fecal virus but does have a significant effect upon pharyngeal virus, then it is conceivable that spread of virus may be diminished by effective vaccination . . ." The evidence supporting the first *if* seems at best equivocal and as regards the second *if*, evidence was at the time entirely lacking.

The New Orleans group[7] in their studies on the spread of virus found no evidence to support the theory that oropharyngeal secretions are more important than fecal excretion where the spread of the virus is concerned. In fact, some of their observations spoke decidedly in favor of the assumption of predominantly fecal spread. Our own observations on the decisive importance of the age of the excreter point in the same direction[2,3]. Transition from the "pre-toilet trained" to "toilet trained age" was associated with a 10-fold reduction in the rate of spread. Furthermore, in a group of about 100 children, virus was detected in the throat in only a few and without any demonstrable correlation with the character of spreader or non-spreader.

It should be remembered, however, that these observations refer to virus strains of extremely low infectivity. The virus concentration in secretions or excretions must surpass a certain critical level to make transmission possible. This happens in the stools in only a fraction of infected persons. In the throat the virus concentration usually is lower by several powers of ten; the minimum oral infectious dose would represent such a volume as to make contact infection mechanically and physiologically impossible. With highly infective strains of virus the situation most certainly is different; in such cases the two modes of transmission should be almost equally im-

portant as far as infection by contact is concerned. However, the possibility of spread through additional channels, sewage, water, and flies would seem always to give fecal excretion an edge. Under such conditions, a reduction in pharyngeal excretion alone could hardly be expected to affect significantly the volume of transmission in the community.

Such an assumption makes poor biological sense. We have to contend that a biological mechanism becomes established only because its acquisition confers a selective advantage. The defense mechanism here discussed would provide protection to other persons but apparently offer no particular advantage to its possessor. If it did exert any selective pressure at all, it would work in the wrong direction.

However, a defense mechanism of the general nature envisaged by Salk does, indeed, seem to exist, although, quite logically, it probably involves the whole of the target area of the virus and not only a limited part of it. Admittedly, present evidence is fragmentary but yet sufficiently suggestive to justify both further studies and some theorizing. Presumably, the presence of antibodies in body fluids, and possibly in secretions, might serve to reduce infection rates by neutralization of virus on the cell surface or even before it reaches the cell. It would also form a kind of barrier against extracellular spread of virus from the primarily infected cell to its neighbours, and thus help in sealing off the original beach-head and limit the volume of primary target tissue involved. This would probably lead to reduction in the amount of virus excreted as well as in duration of excretion.

The effect so far observed is indeed moderate; no reduction in infection rates was observed and a significant shortening of the excretion period to less than 2 weeks in no more than 6% of the subjects, and then only in those with antibody titers well above the average. It is debatable whether this effect is sufficient to explain the radical change in the epidemiologic situation.

However, it should be pointed out that the technic used in the trials mentioned is not particularly well suited to a study of the present problem. The live virus was always given in a dose of 10^6 $TCID_{50}$. Since the maximum virus concentration in the stools after natural infection seems to vary between 10^5 and 10^6 $TCID_{50}$, the dosage of vaccine virus given represents an extremely severe challenge, corresponding to between 1 and 1000 grammes of infectious excretions, far in excess of anything likely to be encountered under natural conditions. It is conceivable, and definitely probable, that, had a more reasonable virus dose been given, a much more differentiated picture would have been obtained. Apparently, this question deserves a more systematic study, including actual determination of the 50%

oral infectious dose and its relation to the degree of serological immunity. Such a study is in preparation.

DISCUSSION

The available evidence taken together seems to justify the working hypothesis that effective immunization with inactivated virus does not only confer protection against paralytic disease but might also produce a relative resistance to oral infection or restrict virus multiplication and excretion, if infection takes place.

The question will inevitably be raised why the Swedish experience seems to differ from that of most other countries, notably the United States. Without pretending to have the right answer I would like to point out that comparative potency tests on domestic and various foreign vaccines, previously reported[8] indicated that the Swedish vaccines possessed a higher average immunogenic potency (for type 1 from 6 to 10 times) and were more uniform. These findings, although disputed by some manufacturers, have been borne out in systematic studies of conversion rates obtained in the field[9] and are also reflected in the surprisingly good protective effect thus far without even a single vaccine failure having been observed in persons given at least two doses.

A look into the Crystal Ball. There are certain reasons to believe that the wild polioviruses might become more or less extinct as the use of vaccines, inactivated as well as live, is extended. Taking the liberty of projecting these speculations into the future, what will happen if and when the classical polioviruses have all but disappeared? Will they leave a vacuum behind, thereby preparing the ground for other viruses, at present, perhaps, more or less suppressed, or will they make way for the emergence of new agents?

Thus far, there is little substance to sustain such speculations. One fact is just emerging, however. As the number of paralytic cases is declining, and at the same time the diagnostic laboratory facilities are improving, more attention can be payed to each individual suspected case, and systematic etiologic studies are now more or less routinely conducted. As a result, a growing number of cases are observed which might previously have been hidden under the diagnosis of poliomyelitis but are now recognized as being of a non-poliomyelitic etiology. Thus, cases of tick-born encephalitis form a distinct group in which only the laboratory can provide a definite diagnosis. Poliomyelitis, like manifestations of Coxsackie A, Coxsackie B, and ECHO-virus infections have likewise been described.[10] Finally there are several groups of cases in which all attempts to find a plausible

etiology have failed to date. In this category are cases of facial paralysis, radiculitis, and polyneuritis, including the Guillain-Barré syndrome and Akureiri disease.

Whether or not such cases are tending to increase in numbers is not yet clear. In any event no spectacular increase has so far occurred. As regards other clinical manifestations of the nonpolio-enteroviruses, such as myopathies, serositis, and aseptic meningitis associated with Coxsackie B, or aseptic meningitis and rubella-like rash found with ECHO-viruses, these manifestations seem to follow an epidemiological pattern much the same as that of poliomyelitis, and independent thereof. One might expect, therefore, that some of the enterovirus infections, now occurring mainly in early childhood and producing insignificant symptoms or none at all, will later reveal themselves as true diseases of civilization and present problems of a similar nature as those associated with poliomyelitis. However, such a development might occur independently of the presence or absence of polioviruses, since it would be entirely a question of the equilibrium between the human host species and the particular type of virus.

It is true that the existence of a certain degree of interference between enteroviruses is probable, and that, therefore, the disappearance of one type might give more room for the others. In view of the great number of types already recognized it is not likely, however, that disappearance of a few members of the group would in any way radically change the ecologic situation. In actual fact, if a hypothetical pressure of interference were reduced, dissemination would be less restricted and the effect, if any, would be in a direction opposite to the one here envisaged, that is, the development towards the state of a disease of civilization would be delayed rather than favoured. Admittedly, the situation could be entirely different if one or more of the poliovirus types interfered more specifically with some particular virus, and especially if the virus did not belong to the enterovirus group. Since it is a purely hypothetical case, however, the idea will not be discussed further in this regard.

On the basis of this reasoning I am inclined to believe that even a complete eradication of the classical polioviruses would not leave any vacuum behind and would not produce any drastic change in the situation as far as other members of the enterovirus group are concerned.

Independently some other viruses such as those of hepatitis, mononucleosis, herpes, and certain of the exanthemata might well be expected to assume increasing importance which would call for specific prophylactic measures. It is advisable to keep a close watch for such a possible development, so that proper action can be taken before the situation becomes acute.

Finally, a few words might be said about the question of new mutants. The accumulating evidence indicates that most of the enteroviruses are sufficiently closely related to be conceived of as derived from a single, or at most, a few ancestral types. For instance, on the basis of present evidence, segregation of the classical polioviruses as a separate subgroup is not justified. They represent rather one extreme of a gradient of decreasing invasiveness and neuropathogenicity for man. If this concept is correct, the large number of types of enteroviruses, although their evolution presumably spans uncounted millennia, is still indication of a definite mutational capacity. There are at present no hints as to the type of selective forces instrumental in the emergence of new types. In such groups as the arborviruses, or above all the myxoviruses, the antigenic relationships make plausible the assumption of a selective pressure of an immunological nature. This is not applicable to the enteroviruses.

Therefore, mass immunization cannot be expected to favour the establishment of new variants of the classical polioviruses or of any other type of enterovirus; so much less so, as the new post-vaccine era will mark a return to the immunological status of the pre-hygienic era, in which the equilibrium between the viruses and their human host was apparently established and maintained without the occurrence of epidemics. With a drastic reduction in the dissemination of virus the chances of mutation will also be proportionally reduced.

To sum up, there seems to be no reason to expect any ominous secondary effects of an eventual eradication of the polioviruses. New diseases will probably appear and existing ones assume increased medical importance in the wake of poliomyelitis, but without causal relation to its disappearance.

The time to rest on our achievements can not yet be foreseen.

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JAMES GEAR*

Poliomyelitis Research Foundation, South African Institute for Medical Research, Johannesburg

COXSACKIE VIRUS INFECTIONS IN SOUTHERN AFRICA

BACKGROUND TO THE STUDY

Systematic studies of poliomyelitis and other enterovirus infections occurring in Southern Africa have been in progress in these laboratories for the past decade. In many respects these studies have proceeded in parallel with the epidemiological investigations carried out by Dr. Paul and his colleagues[1,2,3] in North America, North Africa, and in many islands over the world. They have had a similar purpose—to define the incidence of these infections and the importance of the diseases they cause in this region. In Southern Africa there are clearly defined sections of the population who are distinct in ethnic origin as well as their customs and ways of living. On the one hand there are those of European descent, most of whom live in towns and cities with all the amenities of modern civilization, and enjoy a high standard of living. On the other hand, there are the indigenous African natives, about half of whom still live in their ancestral lands in which they generally retain their ancestral customs. Their drinking water is obtained from springs, streams, and rivers; it is not purified; their huts do not have waterborne sewerage. The other half of the indigenous African population have migrated to the cities and towns to help in the development of agriculture, industry, and mining, where they generally live in sections of the towns set aside for their exclusive occupation. Until recently, the housing conditions in these townships were primitive. Within the last decade, however, most of the African population of the larger cities has been entirely rehoused in model townships supplied, amongst other amenities, with waterborne sewerage and purified water.

It was considered that a study of poliomyelitis as it occurred in these different sections of the population would yield information of value to our understanding of the epidemiology of this disease. The findings of such studies were reported[4] first in 1948 when it was noted that the incidence of paralytic poliomyelitis was about 10 times as frequent amongst those of European descent as amongst the African population. It was also noted that the paralytic cases in the Africans almost all occurred under the age of 5,

* Director, South African Institute for Medical Research.

and the great majority of them under the age of 3, whereas in the Europeans the paralytic cases occurred in all age groups and many in adolescents and young adults. The latter tended to be severe and not infrequently fatal. Serological studies revealed that in all age groups the African population had a higher degree of immunity than the corresponding European group. It was concluded that in his more primitive surroundings the African was more often exposed to infection and so acquired an immunity not shared by those of European descent living in more hygienic conditions. It was anticipated that the provision of better housing with better amenities, better provision of purified water and waterborne sewerage to the African population, would result in an increasing incidence of paralytic poliomyelitis. This anticipation has been realised and the incidence in the African population relative to that in the European has steadily increased. In a recent epidemic in East London, a city with approximately equal numbers in each population group, there were 3 times as many cases in the African population as amongst the Europeans. However, the great majority of the cases among the Africans was in infants.

The trend noted elsewhere in the world of epidemics of increasing extent and severity has thus also occurred in Southern Africa during the last 20 years. In this time there were a series of epidemics, each more extensive, each causing more paralytic cases than the preceding one. This trend was particularly noticeable in the case of the African population and it was anticipated that it would continue unless something could be done to prevent it.

Fortunately, this much needed method of prevention is now available in the poliomyelitis vaccines which have been developed in the last decade. Such vaccines have been produced in these laboratories and mass vaccination campaigns to embrace all the susceptible age groups of the entire population of South Africa have been undertaken.

COXSACKIE VIRUS INFECTIONS

Proceeding in parallel with these studies on poliomyelitis, an investigation of the medical and public health importance of the Coxsackie viruses in this region has been carried out.

MATERIAL AND METHODS

The appropriate specimens were collected from the patients and individuals concerned, and taken immediately to the laboratory. For isolation of virus, suspensions were prepared from the faeces and throat swabs, with penicillin and streptomycin added. Suspensions of blood and cerebrospinal fluid were not treated.

These suspensions were then inoculated as a routine procedure into one litter of one day old baby mice and into two tissue culture tubes of monkey kidney tissue from monkeys *Cercopithecus aethiops pygerythrus*. The litter of baby mice was observed for a period of 14 days for evidence of paralysis, weakness, tremors, and other signs of illness. Mice developing these signs were sacrificed, pieces of the various organs were taken and prepared for histological section and examination. A suspension was prepared from the rest of the carcass for passage into further litters, and when indicated, other studies.

Sections of the organs were microscopically examined for the characteristic lesions of Coxsackie virus infection.

The tissue culture tubes were examined daily for signs of degeneration, and when indicated further passages into other tissue culture tubes were made.

When a virus was isolated in tissue culture, it was first tested against poliovirus antisera. If it proved not to be a poliovirus, it was then inoculated into a litter of baby mice to determine whether it was a Coxsackie virus. If it proved not to be a Coxsackie virus it was given to the ECHO virus team for further study.

The types of poliovirus were identified in tissue culture neutralisation tests. The types of Coxsackie A virus and of Coxsackie B virus were identified in baby mouse neutralisation tests.

OBSERVATIONS

Pathology in Mice. It may be recalled that Coxsackie A virus infections can be clearly distinguished from Coxsackie B infections by the lesions they produce in baby mice. Coxsackie A infections characteristically produce an extensive hyaline eosinophil degeneration and dissolution of the fibres of the voluntary muscles, which is associated with an acute inflammatory infiltration. An outstanding feature of the disease in baby mice is that, apart from congestion of the lungs and of the glomerulae of the kidneys, no other lesions have been described.

Coxsackie B infections characteristically produce an eosinophil degeneration of the brown fat, best seen in the interscapular fat pad. This is associated with acute inflammation and later with calcification of the fat involved. Foci of eosinophil degeneration associated with acute inflammation are often seen in the voluntary muscles. In addition, focal lesions of various organs are often noted. These are most frequent in the liver, pancreas, heart and brain, but may occur in other organs.

Some observations made on the mice inoculated with the strains of virus isolated in the course of this study are possibly worth recording. In a proportion of the mice in which Coxsackie A virus produced typical lesions, it was noted that the thymus was also affected showing degeneration with pyknosis, and fragmentation of the nuclei of the lymphoid cells. These lesions were seen in only some of the mice inoculated. It was presumed that this is a manifestation of Coxsackie A virus infection, but this still has to

be proved and its relationship to Coxsackie A virus infection still has to be clearly assessed.

In the case of Coxsackie B virus infections, it was observed that in mice inoculated with the original suspensions prepared from patients' faeces, the lesions of the fat pad were usually the most prominent features of the pathological picture. Lesions of the liver were not commonly seen but did occur in a proportion of the mice studied. Lesions of the heart were also seen, but their appearance varied from strain to strain and also to some extent depended on the length of time that the mouse survived after it was inoculated. The longer this time the more likely were heart lesions to be found. This was also true of lesions of the brain. These were rarely seen in mice sacrificed before the fifth day after inoculation, but were common in mice surviving for seven days or more. It was noted further that mice inoculated with tissue culture derived suspensions of Coxsackie B virus, often showed extensive, almost complete, degeneration of the liver and extensive involvement of the pancreas at a time when the fat pad lesions were in an early stage of development. In sections of the liver, the cells typically showed margination of the chromatin of the nucleus and pallor of the cytoplasm. Later, the nuclei fragmented and pyknotic fragments of the nucleus were to be seen in shrunken degenerate cells with the eosinophilic cytoplasm. It is possible that this marked involvement of the liver by virus derived from tissue culture suspensions was due to a greatly increased titre of the inoculum.

Clinical Manifestations

It may be recalled that when Coxsackie viruses were first discovered, Dalldorf[6] was careful to emphasize that it was not known what diseases were caused by them. Since that time, studies carried out in various parts of the world have revealed that both Coxsackie A and B groups include important pathogens for Man.

The early investigations carried out in the Department of Preventive Medicine of the Yale University Medical School were reported on by Dr. Curnen[8] at the Second International Poliomyelitis Conference and revealed that these viruses were apparently responsible for some cases of illness resembling non-paralytic poliomyelitis and for cases of short feverish illness, and for Bornholm disease.

Our findings in this field will be briefly noted.

COXSACKIE A VIRUS INFECTIONS

Herpangina. Coxsackie group A viruses have been clearly incriminated as the cause of herpangina by Huebner and his associates[7]. Their findings

have been confirmed in the course of this study and elsewhere. Several cases presenting the typical clinical picture of herpangina were investigated, and members of the Coxsackie Group A viruses were isolated from throat swabs or from specimens of faeces. So far the following types have been encountered in association with this condition, namely Coxsackie Group A 1, 2, 4, 5, 6, 10, and 22. In one case infected with Coxsackie A1 the virus was also isolated from the blood.

Fever with lymphadenitis. In the early summer of 1953, an outbreak of an illness clinically resembling glandular fever was studied in collaboration with Dr. Patz of Middelburg, Transvaal. These patients experienced a sudden onset of their illness and complained of feverishness and of enlarged, tender glands, sometimes associated with painful stiffness of the neck. The fever in the majority of the cases lasted less than 96 hours, but in a few was as prolonged as 10 days. The glands were painful and tender, and enlargement of the spleen was detected in three of the patients. No rash was seen in these cases, but in several patients with a similar condition seen in Johannesburg at the same time, a few roseola spots somewhat like the spots of typhoid fever, were seen on the abdomen. This clinical picture closely resembled that of glandular fever, but the Paul-Bunnell test gave a negative result in 24 out of the 25 patients tested, and the blood picture was not suggestive of glandular fever. Coxsackie A virus was isolated from the stools of several of these patients and from the blood of one of them. The viruses included type 5 and type 6. The virus isolated from the blood was type 5, and its isolation from the blood of one of these patients strengthened the suspicion that it was responsible for the patient's illness and therefore this outbreak.

Hand, foot and mouth disease. Coxsackie Group A16 was isolated from an infant in the longitudinal study carried out in the Germiston African township in 1951. It was not at that time associated with any specific illness. In 1957 in Canada[5], there was an outbreak of illness which was characterised by fever, by blisters in the mouth, and by a maculo-papular rash evolving to a vesicular eruption, involving the hands and feet; the outbreak has been described by Robinson Doane, and Rhodes[6]. A Coxsackie Group A virus immunologically related to A16 was isolated from the faeces of 79% of the cases. Since then, similar outbreaks have been described in England and these have also been associated with infection with Coxsackie Group A16. Several cases of this condition, aptly described as "hand, foot and mouth" disease, have been studied recently in our laboratories. From most of them Coxsackie A virus, identified as Coxsackie A16, in most but not in all cases, has been recovered. One case of particular interest may be noted.

This occurred early in 1961 in a 7-month-old baby who, about 7 days after being vaccinated against smallpox for the first time, developed a vesicular rash, most marked on the hands and feet, but of fairly general distribution. This condition was suspected to be generalized vaccinia, but the vesicles were much finer than the characteristic ones of vaccinia. Coxsackie A16 was isolated from the patient's faeces and presumably was responsible for the patient's illness, including the vesicular rash.

Fever with morbilliform rash. "Fourth" Disease. An outbreak of a feverish illness with a morbilliform rash in which the clinical picture resembled "Fourth" disease combining the features of scarlet fever and rubella occurred in 1958. Several of these cases were admitted to the Johannesburg Hospital under Dr. Arnold Jackson, and in collaboration with him, virus studies were carried out. From a significant proportion of them Coxsackie A4 virus was isolated. The virus was recovered from the blood as well as the faeces of several patients, clearly implying that it was responsible for the patient's condition.

Association with cases of paralytic poliomyelitis. As Melnick[9] and others have found in investigations carried out in the United States, so it has been in South Africa that Coxsackie A virus is often associated with poliovirus in cases of paralytic poliomyelitis. This association may be coincidental, but it occurs so frequently that there is a definite suspicion that these two viruses may, in some way or other, have a synergic effect in producing paralytic poliomyelitis. Whether Coxsackie A virus alone can produce an illness resembling paralytic poliomyelitis is a vexing question. However, from our studies it seems clear that if it can, it only rarely does so, as from the great majority of the cases of paralysis, poliovirus is isolated. However, an interesting observation was made in this regard. From a fatal case of poliomyelitis with bulbar involvement, from which poliovirus was isolated from the faeces, Coxsackie A, type 4, but not poliovirus, was isolated from the spinal cord taken post-mortem. Of course it is possible that the poliovirus which may have been present was neutralised by the patient's antibodies which, at the time of death, had fully developed, and that the Coxsackie A infection was an agonal one.

Guillain-Barré Syndrome. Another condition in which there is still considerable suspicion that Coxsackie A virus may have some role is the Guillain Barre syndrome, a condition which is not infrequently seen in the Johannesburg Fever Hospital where most of these patients are admitted with a diagnosis of poliomyelitis. Many cases occur as post-infective complications of the acute specific fevers, and in this study two cases following chicken pox and one case following Q fever were investigated. Other cases

occur which seem to be related to an auto-allergic or auto-immune reaction to drugs, particularly the sulpha drugs. The aetiology of this syndrome remains uncertain and it is our belief that many cases are not related to Coxsackie A virus infection and have some other basis.

On the other hand, in the course of this study, in a consecutive series of seven cases, Coxsackie A virus was isolated from the faeces of four of them.

Since then Coxsackie A virus has been isolated from a further five cases. In three the isolation was made from the faeces and in two from the cerebrospinal fluid. One of the patients, an adult European woman, died. A Coxsackie Group A type 2 virus was isolated from her cerebrospinal fluid and at post-mortem from the pons and medulla. The other types concerned in these five cases were respectively one A2, one A5 and one A6 from the faeces and one A6 from the cerebrospinal fluid.

These isolations strengthen the impression that some cases of this syndrome are a manifestation of Coxsackie Group A infection. Others may be examples of an auto-allergic hyperreactive state occurring as a post-infective condition.

Meningoencephalitis. Early in the course of this study it was reported[10] that Coxsackie Group A virus was isloated from eleven cases of meningoencephalitis. The same virus was also isolated from the cerebrospinal fluid of three of these cases, a finding which implies that they were the cause of the patient's illness. Later, an additional seven cases from which Coxsackie Group A virus was isloated from the faeces of three, from throat swab of one, and from the cerebrospinal fluid of three.

At that time, in 1955, it was noted that two of the three viruses isolated from the cerebrospinal fluid did not belong to any of the twenty-one serological types then recognised. It was concluded, therefore, that they were a new serological type of Coxsackie A virus. Since then, however, it has been shown that these two strains were Coxsackie A, type 23, better known as ECHO type 9 virus[11]. In the following years it may be recalled that this type of virus was responsible for the most extensive pandemic of aseptic meningitis in Western Europe and North America. Our findings have revealed that this virus is also one of the commonest causes of aseptic meningitis in this region, although extensive epidemics similar to those described in Western Europe and North America have not yet occurred.

In addition to this now well-recognised cause of aseptic meningitis, other types of Coxsackie A virus have been isolated from the cerebrospinal fluid in these laboratories and these include types 1, 2, 4, 14, 16, and 23.

There is little doubt about the aetiological relationship to the condition in these cases. Other types are under suspicion but as the isolation was

made from the faeces and not from the cerebrospinal fluid or blood their role is not yet proved.

The types of virus isolated from the cases diagnosed as aseptic meningitis were as listed in Table. 1.

When infections are as prevalent as Coxsackie A virus infections are, it is difficult to assess the importance of the finding of the virus in the faeces. The finding of the virus in the cerebrospinal fluid and blood is of greater

TABLE 1. VIRUS TYPE ISOLATED FROM CASES OF "ASEPTIC MENINGITIS"

Type	*No. of isolations*
A1	12
2	5
4	8
5	17
6	6
7	3
8	4
9	4
10	2
11	2
16	1
17	2
18	3
22	5
23	18
24	1
Total	93

significance. It is therefore worthwhile to note that during this period several types of Coxsackie A viruses were isolated from the blood of the patients concerned (Table 2).

It may also be of interest to note that, in addition to Coxsackie A9 and A23 (ECHO 9), primary isolations of several other types were made in tissue cultures inoculated with the original suspensions from patients (Table 3).

COXSACKIE GROUP B VIRUS INFECTIONS

Early studies reported by Dr. Curnen[6], and carried out by the workers in Dr. Paul's department at Yale University, incriminated Coxsackie Group B virus as the cause of Bornholm disease and also suggested that the in-

fection often gave rise to a feverish illness with pharyngitis and to an illness resembling non-paralytic poliomyelitis or aseptic meningitis. These findings have been confirmed all over the world and in this region epidemics of Bornholm disease have been related in turn to Coxsackie Group B2, B3, and B4 viruses.

TABLE 2. TYPES OF COXSACKIE A VIRUS ISOLATED FROM BLOOD

Type	*No. of isolations from blood*
Coxsackie A1	1
" A4	4
" A5	10
" A9	1
" A10	3
" A22	4
" A24	1

TABLE 3. TYPES OF COXSACKIE A VIRUS ISOLATED IN TISSUE CULTURE

Type	*No. of primary isolations in tissue culture*
Coxsackie A1	1
" A2	2
" A4	14
" A5	5
" A6	1
" A7	2
" A10	1
" A16	1

However, it is not only as a cause of Bornholm disease that these virus infections have attracted attention in this country. Studies carried out in these laboratories have shown that Coxsackie Group B virus is one of the commonest causes of meningoencephalitis in this region. Similar cases occurring in Rhodesia have also been found to be associated with Coxsackie Group B virus infection. In several of these cases, weakness or even paralysis of some groups of voluntary muscles was noted. However, in all of them the condition was transient and no permanent paralysis resulted.

A tendency to mutual exclusiveness of Coxsackie Group B and polio-virus infections in contrast to the common association between Coxsackie

A and poliovirus, has also been noted here. However, this exclusiveness is not absolute, and in several cases of paralytic poliomyelitis from which poliovirus was isolated from the faeces, Coxsackie Group B virus was also isolated. One of these patients died of paralytic poliomyelitis.

In the course of this study Coxsackie Group B virus was incriminated as an important cause of *Myocarditis neonatorum*. The first evidence incriminating this virus as a cause of encephalomyocarditis in newborn babies was given by Gear and Measroch[11, 12], who studied an outbreak which occurred in a Maternity Home in Johannesburg in October and November of 1952. Ten newborn babies became ill while still in, or soon after their discharge from, the Maternity Home in which they were born. They had fever and developed signs and symptoms of acute myocarditis; in some cases, signs of meningoencephalitis. Four recovered and apparently have suffered no serious sequelae. Six died after an acute fulminating illness ending in circulatory collapse[13]. Post-mortem examination carried out in three of them revealed that the cause of death was acute heart failure resulting from a focal but extensive myocarditis. In addition, in one case a focus of inflammation in the brain and of one suprarenal gland was found, suggesting that the myocarditis was a part of a generalized infection of which the heart bore the brunt.

Shortly after this, in December of 1952, another outbreak involving three babies occurred in another Maternity Home. These babies all showed, in addition to fever and marked tachycardia suggestive of myocarditis, signs of meningoencephalitis including a pleocytosis of the cerebrospinal fluid. All recovered, and from the faeces of the only one examined, Coxsackie B3 virus was isolated.

A third outbreak occurring in a Maternity Home in Umtali, Southern Rhodesia, was investigated, in collaboration with Dr. Montgomery[14]. In this three newborn babies were affected. Two recovered after being ill about one week and a third died on the 12th day after birth. On post-mortem examination the heart showed focal myocarditis. A virus identified as Coxsackie Group B was isolated from the faeces and the caecal content of the baby who died, and from the faeces of one of the babies who recovered.

In 1955, Coxsackie B type 2 virus was isolated from the heart muscle of a 9-day-old baby who died soon after admission to the Transvaal Memorial Hospital for Children with fever and signs of myocarditis. Sections of the heart showed an extensive focal myocarditis similar to that described in the previous cases. The relationship of Coxsackie Group B virus to the condition was thus proved.

The aetiological role of Coxsackie Group B virus in causing this disease, often fatal in newborn babies, was convincingly confirmed in 1955 by workers in Holland[15] and the United States. In 1956 Kibrick and Benirschke[16] described a fatal illness in a newborn infant from whom Coxsackie Group B type 3 virus was isolated from the spinal cord. More recently, Enders and his co-workers[17] in Boston have reported briefly on the isolation of Coxsackie Group B4 virus from a similar case. Since the first outbreak occurred in 1952, a number of similar cases have come to our notice. It is clear that this is an important hazard of the neonatal period.

WAVES OF INFECTION

In addition to incriminating Coxsackie viruses as the cause of a number of prevalent or important conditions, it also became apparent during this study that the infections of the different types occurred in waves. This became clear from a longitudinal study of a group of 20 Bantu babies in the Germiston African Township begun in 1948 to determine the prevalence of poliovirus and other enterovirus infections. This study was continued for five years and during this time all the then known types of Coxsackie A virus except A13, and all the then known types of Coxsackie B virus except B1 were isolated. In addition, several new serological types were isolated and their identification as new types was confirmed by Dalldorf and Sickles. Among these was A16 which six years later was incriminated as the cause of "hand, foot and mouth" disease.

The occurrence of waves of infection was also apparent from the isolations made from cases of illness. This was particularly clear in the case of Coxsackie B infections. In 1951-52 a wave of B3 infections occurred. In 1954 a wave of B4 and in 1956-57 a wave of B2. This was followed by another wave of B3 in 1959. At no time in this period did a wave of B1 infections occur. It was therefore with considerable interest that we noted, in 1960, the appearance of B1 virus for the first time since the study began in 1948. The first isolations were made from specimens received from Durban and Bulawayo in July, the next from Nairobi, Kenya, and Salisbury in Rhodesia in August. Four isolations were then made from patients in Johannesburg in September.

OBSERVATIONS MADE DURING A RECENT EPIDEMIC OF COXSACKIE B1 INFECTION

It was anticipated that a widespread epidemic would occur. This anticipation was realised and the most extensive epidemic of Bornholm disease so far recognised in this area occurred in the summer of 1960-1961. The features of this epidemic will be briefly noted.

There were many hundreds of cases, but virus isolation was attempted on only a few of those admitted to hospital. However, the curve of the epidemic is reflected in the number of isolations made each month (Table 4).

It was clear from these as yet incomplete typing studies that nearly all the cases investigated were due to Coxsackie B1 virus, but it is of interest to note that a few B2 infections also occurred in this period.

TABLE 4. ISOLATION AND TYPES OF COXSACKIE B VIRUS IN THE 1960-1961 EPIDEMIC

	No. of isolations	*Types so far identified*	
1960		B1	B2
September	4	3	
October	14	11	1
November	69	61	1
December	62	49	
1961			
January	33	22	2
February	15	7	1
March	16	6	
April	5	3	
May	3	Not done	
June	2	" "	

CLINICAL MANIFESTATIONS

Examples of all the well-known manifestations of Coxsackie B infection were seen in the cases admitted to the Johannesburg Fever Hospital and the Transvaal Memorial Hospital for Children.

Bornholm Disease or Pleurodynia. Most cases presented signs and symptoms of this condition. It was again noted that in adults, pains in the chest were more frequent than in children who more often complained of abdominal pain. Many cases were admitted to hospital diagnosed as acute appendicitis, and some were operated on for this condition, fortunately with no ill effects.

Aseptic Meningitis. Many cases of this condition were admitted to hospital and proved to be due to Coxsackie B1 virus by its isolation from the cerebrospinal fluid.

Myocarditis and Pericarditis. Soon after the isolation of the first Coxsackie B1 viruses from cases in Johannesburg, the Medical Officers of all

the towns and cities of the Witwatersrand were warned that an extensive epidemic of this infection was anticipated. It was suggested that precautions should be taken to prevent the introduction of the infection into Maternity Homes. Such precautions were then taken in all centres. These consisted of allowing only the husband to visit mothers confined to the Maternity Homes. All other visitors were debarred. Patients developing a febrile illness were isolated; nurses developing an illness possibly caused by Coxsackie B virus were put off duty for one month after the onset.

In spite of these precautions the infection was introduced into all the large Maternity Homes in Johannesburg. In most cases the source of the infection could be traced to a recently admitted expectant mother or to a nurse. About twenty cases of *Myocarditis neonatorum* were brought to our notice and investigated.

Since the Maternity Homes had been alerted to the hazards of this infection, it was possible to isolate the patient and the mother and to close the affected ward. It was kept closed until all the inmates had been discharged and the ward had been disinfected. In each instance these precautions prevented further spread of the infection and there were no major outbreaks such as the one investigated in 1952 when six of ten babies died. Of the twenty cases, three died.

In addition to infection in newborn babies, cases of myocarditis were seen in older infants and in children. There were in addition several in adults, usually associated also with pericarditis. One of these patients, a member of the staff of this Institute, developed circulatory failure. It seemed likely that this collapse would have had a fatal outcome except for the fact that he was in hospital where immediate measures were taken to tide him over the critical period. After a prolonged convalescence he has fully recovered. No fatal cases were noted in older children or adults.

This epidemic was of interest in that its occurrence was clearly forecast from the knowledge gained over the past decade, knowledge gleaned from data on the Coxsackie virus infections in this region. It emphasized the value of keeping a check on the state of immunity of the population.

The outbreaks in Maternity Homes also emphasized the need for protective measures. It appears that the development of a prophylactic vaccine against the Coxsackie B viruses for selective use to protect non-immune mothers would be a worthwhile undertaking.

FUTURE TRENDS

The development of prophylactic vaccines and the perfection of other methods of prevention also appear desirable for a number of other reasons.

It is believed that the trends, which became so clear in the epidemic patterns of poliomyelitis since the beginning of this century are being, or will be, followed by the emergence into greater prominence of other alimentary virus infections. Until recently, it seemed likely that most of these infections occurred in infancy and early childhood and contributed to the large number of mild feverish illnesses experienced during this period of life. Indeed, this has been shown to be so in a number of longitudinal studies. It may be anticipated, however, that as hygienic conditions are improved there will be a tendency for the infants to escape and for the brunt of the infections to be borne by the older age groups.

When this happens, it is probable that the clinical manifestations will become more severe. They are particularly liable to be severe in adults. The proportion of adults who, because of lack of earlier infection will remain susceptible, will probably progressively increase. In time, the proportion of women of child-bearing age who lack immunity to important pathogens of this group of viruses will also increase. Whether infection by any of these viruses contributes to foetal death or malformation is not known. It is also possible that, in addition to the Coxsackie B viruses, some of the Coxsackie A viruses and other enteroviruses infecting babies born of mothers lacking immunity may give rise to severe, possibly fatal illness. These possibilities, and indeed the whole field of these virus infections, are in need of further investigation. The studies so far carried out, many of the most significant in or under the aegis of Dr. Paul's Department at Yale University, suggest that this continued exploration will be most rewarding.

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W. MCD. HAMMON*

Department of Epidemiology and Microbiology, Graduate School of Public Health, University of Pittsburgh

GLOBAL IMPORTANCE OF THE ARTHROPOD-BORNE VIRUSES AFFECTING MAN

It has been pointed out previously[1] that the arthropod-borne (arbo) viruses affecting man are principally, and possibly all, classifiable as zoonoses and that man is an accidental and frequently a terminal host in the normal biological cycle of the agent. This concept, although important from the standpoint of epidemiology and control, tends to be misleading in respect to the importance of the human disease problem. In certain areas infection rates in the non-human vertebrate and in the arthropod vector become so extremely high that the spill-over to man by way of vector feeding on man assumes major significance. This may happen annually or sporadically. Furthermore, a number of these agents in addition to their basic, reservoir cycle in nature can, under special circumstances, be transmissable from man to highly domestic, anthropophilic vectors which, in turn, transmit directly and essentially only to other human hosts. This short, relatively direct cycle may result in extremely explosive and catastrophic epidemics. Examples of viruses in this latter group are those causing yellow fever, dengue, phlebotomus fever, chikungunya,[2,3] O'nyong-nyong or Gulu[4,5] of Africa, and those of the hemorrhagic fevers of the Philippines and Thailand.[6–8] Still another type of epidemic situation is recognized in many countries in Europe and the eastern USSR in which goats which have become infected with members of the Russian tick-borne encephalitis virus group excrete the virus in the milk and thus may simultaneously infect small or very large groups of persons consuming the milk.

Arbo viruses may be classified in a number of ways. Antigenically, mainly on the basis of hemagglutination inhibition, they are currently classified by Casals[9] into several large and small groups with a relatively large heterogeneous unclassified group left over. Within each group of related agents the viruses are distinguished one from the other by means of either the hemagglutination, the neutralization, or the complement fixation test. This classification, however, is of particular importance only to those engaged in laboratory-related aspects of the problem. The clinician finds that these viruses may cause any of several different syndromes, including encephalitis,

* Professor of Epidemiology and Microbiology.

dengue-like systemic febrile diseases, hemorrhagic fevers, and a few other peculiar entities.[9,10] The epidemiologist and the public health personnel responsible for control must most frequently classify the problems created by these viruses by the type of vector or by other epidemiologic factors connected with transmission to man and maintenance of the virus in a basic biologic reservoir. This is done most conveniently first by the vector, i.e., tick, mosquito, phlebotomus, and possibly mite. Each of these may then be subdivided on the basis of the vertebrate species from which they derive their infection, whether birds, rodents, large mammals, or man. Next of importance in epidemiologic considerations comes the type of climate where the disease problem appears. In tropical areas transmission may occur at all seasons and a man-arthropod-man or other cycle may go uninterrupted, while in temperate climates the agent disappears under conditions of a strictly man-arthropod-man cycle during seasons when the vectors are inactive. The virus must be re-introduced if disease is to occur again. Under such circumstances a reservoir mechanism for carrying the virus through the winter is necessary if an endemic situation is to exist. Many of these reservoirs are as yet poorly recognized.

About two years ago the author, with the assistance of others, summarized in tabular form the viruses then recognized as producing disease in man.[10] They were presented in a combination of clinical and epidemiologic classifications, together with the type of vector. At that time there were 45 such viruses recognized as causes of human disease. Probably more than a dozen additional ones have been recognized subsequently. Work[11] has recently counted 51 such viruses. There are at least 75 other viruses isolated and identified which are either known to infect man or suspected in this category, but the disease they produce, if any, has not yet been recognized with certainty.

In this short review only a few examples will be used to illustrate the recognized and potential importance of this group of diseases.

MAN-VECTOR-MAN CYCLE

Yellow fever

The importance of this disease in the past needs but brief review. Such review, however, should serve as a reminder that what happened before a large degree of control was effected can happen again, should control be relaxed, or can occur in new areas without control, should the virus be introduced. Yellow fever serves as an excellent example of the potentiality of all those members of the arbo virus group in which the virus circulates

in the blood of man and can thus infect highly domestic arthropods with a preference for human feeding.

This virus is transmitted in urban areas by *Aedes aegypti*. They breed principally in and near houses in domestic water containers and they feed and rest in houses. Under these circumstances the spread of infection from a single case or a single infected mosquito is usually explosive in character and if not arrested early by energetic mosquito control leads to morbidity rates which reach exceedingly high levels, and case fatality rates up to 40 per cent. Terrible epidemics struck repeatedly at many North American coastal cities, in cities in Latin America, and in Africa. Yellow fever played an important role in history through the digging of the Panama Canal in that the canal would have been controlled by France except for the impregnable resistance offered by this disease. Yellow fever yielded subsequently to Americans when the vector was recognized and control undertaken.

Research of a long, expensive, and arduous nature, which was associated with a number of fatalities among the pioneer scientists, slowly led to identification of the cause, specific diagnostic tools and survey methods, determination of vectors in many areas, their ecology, and methods of control in urban areas. Then came the discouraging finding of forest reservoirs of infection in monkeys and tree top mosquitoes; control under these circumstances required development of a vaccine. But to develop a vaccine was a major research project culminating in selection of avirulent strains and extensive field trials for safety and evaluation of effectiveness. As recently as World War II when the vaccine problem was considered solved, serum hepatitis virus as a vaccine contaminant became another serious set-back and required further research before human serum was eliminated as a vaccine constituent.

Even with the lessons learned from the development and evaluation of yellow fever vaccine, control of the large number of other arbo viruses by vaccine will still require major effort. As of today not a single other live, avirulent vaccine is available beyond the experimental stage. Fortunately, progress is being made with several, but each presents a unique and exceedingly difficult problem.

In recent years yellow fever has presented new alarms. The jungle infection has spread northward through Central America into southern Mexico and very costly efforts of surveillance and curtailment have been undertaken. Discovery of yellow fever in a port city in Trinidad produced international reverberations and economic crises. Ethiopia and the Sudan are also scenes of recent and current concern.

India, Pakistan, Indo China, Indonesia, China, the Philippine Islands, and many other areas of the Far East where the vector has long been recognized have, for some unknown reason, never experienced this disease. There is no recognized reason why, if suitably introduced, it would not result in one of the great disasters of history. For this reason and for the protection of other potential areas where the vector is permitted to breed there have been repeated international conferences on notification and control. The WHO is now sponsoring these services but many governments are very active in enforcement of vaccination requirements, plane and ship disinfestation, *Aedes aegypti* eradication or control, etc.

Dengue, Chikungunya, Mayaro, O'nyong-nyong, Philippine, and Thailand Hemorrhagic and Phlebotomus fevers.

This is another group which like yellow fever has anthropophilic vectors, frequently *Aedes aegypti,* and the virus may be obtained directly from other human cases. In fact, other vertebrate hosts are strongly suspected of serving as reservoirs, as in jungle yellow fever, but this is yet to be established. Field research along these lines is seriously needed.

Dengue viruses are much more wide-spread in distribution than yellow fever, are equally explosive, and cause equally high morbidity rates but, fortunately, are rarely associated with mortality. Nevertheless, dengue is often seriously debilitating and may be followed by a prolonged convalescence. It has nearly immobilized many urban communities and in times of war it has completely destroyed the effectiveness of whole battalions. Chikungunya and O'nyong-nyong viruses in the last few years have caused similar havoc in several areas of eastern and central Africa. They produce a disease quite similar to dengue but with a greater tendency toward severe arthritic manifestations. Mayaro virus of Trinidad and South America has a somewhat similar clinical and epidemiologic pattern.[13,18] Phlebotomus fever of at least two types has produced large scale and seriously debilitating epidemics in the Mediterranean area, the Near East, and parts of the U.S.S.R.[14] This became an important military disease during World War II causing great concern to Americans, their allies, and to the Germans. The phlebotomus vectors are again principally domestic. Conversation with Russian scientists, which the author was privileged to carry out in the U.S.S.R. recently, revealed that they have evidence of wild phlebotomus and wild vertebrates maintaining a reservoir in remote, uninhabited areas. Susceptible humans passing through such an area or briefly camping there may become infected. Again, it is fortunate that this disease, although a serious problem, does not have important mortality associated with it.

The present section might end on this somewhat benign theme if it were not for events in the Philippines and Thailand which were investigated initially in 1956. Epidemics of hemorrhagic fever began to occur in small children in Manila during the rainy season of 1954.[15] A major outbreak of over 750 hospitalized cases with a 5% to 10% case fatality rate was investigated in 1956.[16,6] Two new types of dengue virus (named types 3 and 4) were found as etiologic agents.[7] Type 3 was isolated from *Aedes aegypti* during the epidemic and experimental mosquito transmission was effected to laboratory animals by this same mosquito. All epidemiologic circumstances incriminated *Aedes aegypti* as the vector. Repeated outbreaks have occurred in other urban areas of the Philippines in subsequent years. This new disease in the dengue family associated with a very significant mortality assumes, therefore, the serious characteristics of yellow fever. Fortunately, it has occurred in a population where immunologically related dengue virus infections were endemic and all the older and much of the younger segment of the population was immune!

In 1954 a rather similar, although distinct, disease occurred in epidemic form during the rainy season in Bangkok, Thailand. A much more serious epidemic occurred there in 1958.[8] Over 3,500 cases were hospitalized from the urban area and there were over 300 deaths. The etiology again proved to be mixed, and there is still much work required to clarify the complex picture. Two viruses were repeatedly isolated from the acute phase blood of patients, chikungunya virus or one antigenically essentially identical, and a virus first tentatively identified antigenically as dengue type 2.[7] However, the latter, on further antigenic study may possibly be classified as dengue type 5. Still another dengue virus was isolated once[17] and other agents continue to be under study. Serologically, the majority of patients could be diagnosed as infected by a virus of the dengue group or by chikungunya virus, and many developed rising antibody titers to both viruses. A few did not respond with antibodies to either Group A or B arbo viruses.

In Bangkok dengue virus of the predominant type found in patients was repeatedly isolated from *Aedes aegypti* while chikungunya virus was isolated from *Culex quinquefasciatus* (*Culex fatigans*). As in Manila, the disease was limited to young children and epidemiologic investigation incriminated domestic mosquitoes, principally *Aedes aegypti,* as the vector. Each subsequent year has brought recurrent epidemics of the same disease to Bangkok and adjacent urban areas, and frozen sera sent from Bangkok to the author's laboratory by Dr. Ethel R. Nelson during 1960 have again led to confirmatory virus isolations and serology indicating activity of these

two separate and distinct viral agents. Again, one must consider the potentialities should this virus infection occur where the population is not largely immune as a result of previous infection with similar or closely related agents which happen to be far more benign. The situation is equally serious when large numbers of non-immunes are required to move into an endemic area as a result of war or political crisis. Such may well occur in the near future.

A recent outbreak of a rather similar new disease has just been described in a preliminary manner from Singapore and a dengue-like agent is suspected[18] as the cause.

These hemorrhagic fevers are definitely recognized as *new* diseases in these areas and we may well expect them to appear in other areas where the common vector mosquitoes are prevalent. They have already become major epidemic problems in Bangkok, competing in morbidity and mortality with outbreaks of cholera; the two diseases alternate in different seasons of the year, hemorrhagic fever during the rains and cholera in the dry season.

ZOONOSES

Many of us still recall vividly the first great outbreak of human encephalitis of the arthropod-borne type which occurred in St. Louis in 1933. Newspapers over the whole United States carried almost daily headlines of the increasing numbers of cases and deaths. In retrospect it appears that an unusually dry summer led to stagnation of many polluted streams and drainage ditches giving rise to intense breeding of *Culex quinquefasciatus,* the vector. Subsequent epidemics in the same and many other areas have been of less magnitude, although with equal or greater morbidity rates. However, it must be recalled that given suitable conditions epidemics equal to or greater than that of St. Louis can occur.

The author began studies of a series of annual encephalitis epidemics of western equine and St. Louis types in the Yakima Valley of Washington in 1940.[19] This was the third consecutive year of demonstrated viral activity in the area and the disease continued to occur in a similar severe epidemic pattern for several years more.[20] This was of major concern to this highly agricultural community which bloomed in a desert, thanks to irrigation. The very irrigation, however, was responsible for the tremendous numbers of *Culex tarsalis* found to be the vector of both viruses. Control came by accident when DDT replaced other less effective insecticides used strictly for the control of agricultural insect pests.[20]

The author also was privileged to study the ecology of these two viruses over periods of high and low endemicity in the San Joaquin Valley of

California.[21] Another desert area which had been turned into an agricultural paradise through irrigation began to face serious difficulties through the constant morbidity and mortality from these severe encephalitic diseases. Many children with serious mental and motor residual from infection during infancy were admitted to the mental and chronic disease institutes of the state. Only after years of research on the epidemiology and ecology of the vector, and millions of dollars of local and state tax funds were devoted to organized *Culex tarsalis* control, did a reasonable degree of control occur. Even this broke down in the summer of 1952 when the largest epidemic of the western equine type ever to occur in the area resulted from unusual snow fall in the mountains during the previous winter.[22] Floods and wasteful irrigation practices resulted in an unprecedented and overwhelming bumper crop of *Culex tarsalis*. Fortunately, control was established before St. Louis virus, which normally follows after the peak of the western equine disease, had become established in epidemic form.

The greatest epidemic year for Western equine virus (1941) will never be forgotten in Montana, the Dakotas, and Manitoba where thousands of cases occurred. This came unexpectedly in dry farming country when climatic and meteorological conditions combined to produce an unprecedented number of vector mosquitoes. The tragedy was so great that a large human vaccination program was attempted the following year in Canada, using the vaccine previously prepared for horses.[23] This type of epidemic can be expected to recur when conditions are optimal unless preventive measures, not now considered practicable or economically feasible, are carried out. In addition, rapidly developing irrigation in many new areas of the West is posing potential problems.

All of us in the United States have recent awareness of the relatively minor epidemic of eastern equine encephalitis which occurred in New Jersey in 1959. This was serious from the standpoint of hysteria and economics in a summer resort area. When such an epidemic occurs in crowded East Coast areas, as in the one just mentioned as well as the one in Massachusetts in 1938, it assumes major importance even though morbidity is quite low. These outbreaks again appear in rather unpredictable fashion apparently from a concurrence of factors leading to a high population of *Culiseta melanura* and possibly other vectors. Much more must be learned before these epidemics can be aborted in the early season as a result of adequate biological surveillance.

Japanese B encephalitis, although little known to physicians in this country, has been of far greater major epidemic importance than any of the encephalitides so far discussed. Americans encountered it first on

Okinawa in 1945 and further felt its demoralizing and fatal effect during many years of occupation there, in Japan, in Korea, and on Guam. It is prevalent and of great importance throughout much of Asia from the Maritime Provinces of the U.S.S.R. through Indo China and into India. The American experimental vaccine available for use in Okinawa in 1945 proved inadequate through several years of use at a cost of many millions of dollars. No vaccine is available yet, although in our laboratory an attenuated strain is showing promise in animals. The author, after observing several hundred cases of the Japanese disease in numerous areas, cannot exaggerate the importance of this crippling and demoralizing disease which can kill thousands in a season and create hysteria like few other diseases through the horrors of its clinical manifestations and residua.

The many forms of Russian spring-summer encephalitis, rarely heard about in our country and seldom seen by American physicians, cannot be rated second even to Japanese encephalitis. They occur almost continuously in sporadic epidemic or endemic form in at least thirty recognized areas of the U.S.S.R. and in a number of other European countries including part of Sweden. In the U.S.S.R., the development of new areas in wild forest lands is associated with a very high morbidity among the new settlers and workmen. Residual paralyses are very common and the mortality of the eastern form is high. A formalin treated mouse brain vaccine has been used on millions of persons on an annual basis for a number of years. The vaccine admittedly has its own morbidity in complications, but the severity of disease and the high incidence render its use imperative until a safer product can be devised. In that country tremendous effort has been given to this important problem and the author has just had an opportunity to visit with a number of scientists devoting their total energies to this pressing need. Deaths and crippling have occurred frequently among those studying this disease and the staff of the author has not escaped unscathed.[24] In addition to infection by mouth through milk, aerosol infection in the laboratory has posed a serious problem.

A review of the arbo viruses would be incomplete without mention of Kyasanur Forest disease in India, and Murray Valley encephalitis in Australia, as well as a number of others, but it is hoped that examples presented, many of them from some first-hand experience of the author, will have served the intended purpose of pointing out to those not living in threatened areas, or to those conscious only of a local situation, that a tremendous global problem exists.

The day of conquering important infectious disease problems, claimed by many as having arrived, is still far off. In fact, we probably recognize

only the fringes of the arbovirus disease problem. Control, except for yellow fever, is quite remote even for a few of the viruses which have been studied most. The complex epidemiological and virological problems call for team field investigations as well as local and central laboratories staffed by a large and complex group of physicians, epidemiologists, veterinarians, mammologists, ornithologists, entomologists, ecologists, virologists, and immunologists. These must work in a coordinated fashion throughout the world, exchanging information and virus strains, identifying viruses, and developing and exchanging new techniques. A beginning toward this has been made by an American pioneer group in a series of organizational meetings. A loose-leaf virus catalogue has been assembled and a news letter printed and exchanged by participants. This has been accomplished with assistance from The Rockefeller Foundation. Plans to establish and extend this type of collaboration and exchange have been under way by the WHO and several meetings have been held, but funds are not yet available to launch this program as planned. It is to be hoped that the needed personnel can be recruited, trained, and supported for this important task force to work throughout the world. Tentative planning through the National Institutes of Health may help provide necessary but exceedingly costly identifying reagents. These are imperatively needed by many workers now struggling to produce by themselves the hundreds of different reagents required to identify the more than 100 agents already recognized.

Since there is no national foundation for arbovirus diseases in the United States and Congress has not yet recognized the importance of this problem as equal to that of many of the non-infectious, chronic disorders for which specific institutes have been established, work in this country will have to continue to compete vigorously with all other infectious disease problems for support. It is to be hoped that this brief review will help to spark a world-wide as well as an increased domestic program.

It is certain that Dr. John Paul, in whose honor this issue of the Journal is published, supports these views in full. His laboratory has played an important part in the study of certain of these diseases in past and recent years and his personal interest has covered several decades.

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W. PAUL HAVENS, JR.*

Department of Medicine, The Jefferson Medical College, Philadelphia

VIRAL HEPATITIS†

FOREWORD

The privilege of studying a disease over a span of almost two decades is an unusual one. However, it is quite clear that an important factor enhancing the value of the experiences cited above was the opportunity of working with a rather closely knit group whose interests and ideas were intimately shared. Throughout the years, Dr. John R. Paul found himself the central figure in this group; the influence of his quiet but unquenchable enthusiasm has been, and is, an abiding strength during the numerous frustrations. It was his responsibility to suggest when to alter the direction of current investigations as often as it was to initiate or encourage new ones; indeed, his perception and unerring sense of timing saved the day on more than one occasion. It was he who in various parts of the world returned again and again to a favorite pastime — the pursuit of clinical epidemiology — and thereby clarified some of the problems of hepatitis. In retrospect, it is no accident that this disease should have taken its place among his dominant interests which include so many others, such as scarlet fever, rheumatic fever, infectious mononucleosis, and poliomyelitis.

INTRODUCTION

The physicians and, in fact, all the people of this country have been made painfully aware of the importance of viral hepatitis by reason of the large military and civilian epidemics that have occurred here and in various other parts of the world during and after World War II. These epidemics were primarily concerned with infectious hepatitis (Virus A), although a notable exception was the huge outbreak of serum hepatitis (Virus B) in United States troops following their inoculation with yellow fever vaccine (Table 1). Recognition of the rapidly growing role of

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* Professor of Medicine, The Jefferson Medical College. Formerly Research Assistant (Assistant Professor), Section of Preventive Medicine, Yale University School of Medicine, 1945-1946.

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the latter form of disease has evolved both here and abroad during the past two decades as a natural sequence to the increasing opportunities for artificial transmission of hepatitis viruses. At a meeting of the Expert Advisory Committee on Hepatitis of the World Health Organization in Belgium in 1952, cognizance was taken of the international impact of both forms of disease and the problems they presented.[1] The introduction of our medical profession, hitherto curiously naive in its appreciation

TABLE 1. MORBIDITY PER 1,000 AVERAGE STRENGTH PER YEAR FOR VIRAL HEPATITIS (INFECTIOUS HEPATITIS AND SERUM HEPATITIS) AMONG U. S. ARMY PERSONNEL, 1942-1953*

Date	*Total Army*	*EUCOM*	*FECOM*	*Korea*	*Cont. U.S.*
1942	12.6	21.5	23.0		10.3
1943	3.6	6.8	2.8		0.6
1944	4.0	2.6	9.1		0.7
1945	11.1	9.4	43.8	6.4**	2.6
1946	4.5	6.7	6.4	5.3	3.1
1947	4.0	9.1	3.2	3.1	2.5
1948	3.7	12.7	3.8	6.0	1.8
1949	3.4	12.2	3.8	7.7	1.4
1950	4.5	12.3	10.4	19.0†	1.2
1951	4.9	7.8	13.7	16.9	1.4
1952	3.6	5.0	6.3	6.0	2.1
1953	2.7	3.6	4.3	3.7	1.7

* These data were obtained from summary reports and are to be considered provisional.

** Includes data for September-December 1945 only.

† Includes data for July-December 1950.

of viral hepatitis, was a rude but forceful one and was brought about by the occurrence of some 200,000 cases in United States troops from 1942 to 1945. That we were not instantly prepared to meet this unexpected situation might be partly explained by the facts that (i) this was the first time that serum hepatitis had appeared as an important military disease and (ii) the paucity of epidemic hepatitis in our troops in World War I had not directed our attention to its possible importance, this despite our historical background of 71,691 reported cases of jaundice among white troops in the Union Army in the Civil War. It is, however, a tribute to the energy and sagacity of military medical officers and their civilian colleagues that a realistic appreciation of the problems soon came about.

EPIDEMIOLOGY

Viral hepatitis has, in general, been a belated entry in the lists of reportable diseases, although the cyclic recurrences of large epidemics at irregular intervals of five to ten years or longer have been well documented in many places in the world. Sweden and Denmark were pioneers in the recognition of both forms of disease and in their epidemiology. The statistical records of the latter country during the past thirty-three years provide the longest and most accurate uninterrupted history of experience

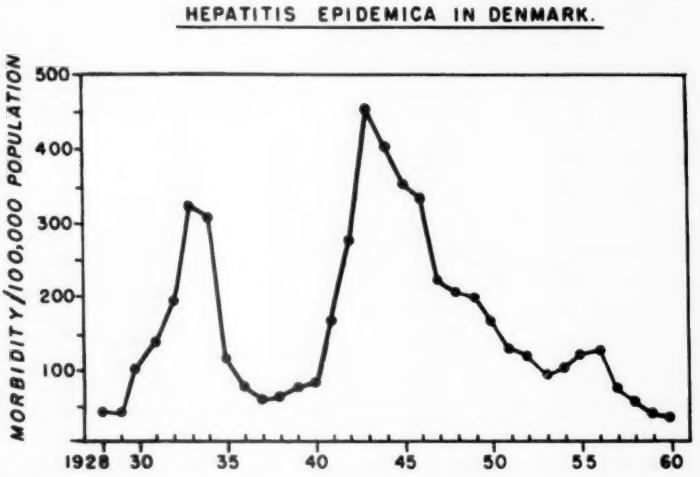

FIG. 1. These data were kindly supplied by Miss Marie Lindhardt, Chief of the Statistical Section, the National Health Service of Denmark.

with epidemic hepatitis, which was made notifiable in Denmark in 1928. From that time through 1960, 210,779 cases were reported. The rough similarity of this figure with the number of cases reported in United States troops in the short period 1942-1945 emphasizes the vast military importance of the latter experience. In Denmark, two major epidemics occurred; the first beginning in 1930 and reaching its peak in 1933, the second beginning in 1941 and reaching its peak in 1943 (Fig. 1). It was during this second, larger, outbreak that an unprecedented and as yet unexplained sharp increase in mortality from liver disease occurred (18.7—21.4 per 100,000 population), reflecting mainly the great increase in lethality of hepatitis, particularly among older women (Fig. 2). Speculation on the cause of this was largely concerned with factors in the host;

however, there is some reason to suspect that changes in virulence of virus might also be operative. Similar unexplained temporary increases in lethality have been recorded in other countries. It is of interest that this highly fatal form of acute hepatitis has largely disappeared from Denmark, and the mortality has ranged from 0.2—0.7 per 100,000 population for the past several years. That the mortality from liver disease

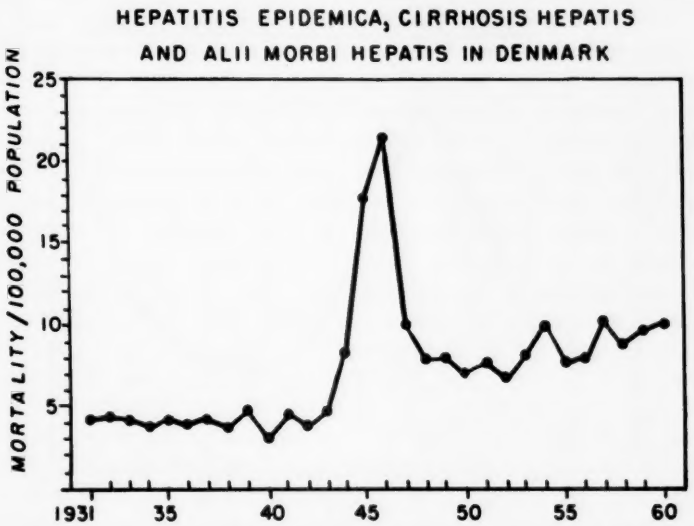

FIG. 2. These data were kindly supplied by Miss Marie Lindhardt, Chief of the Statistical Section, the National Health Service of Denmark.

in Denmark in the years following the second epidemic has gradually increased and remained at a substantially higher level, reflects largely the increase in deaths due to cirrhosis. How many of these cases developed as a sequence to hepatitis is undetermined, although it is likely that this was an important contributing factor. Bjørneboe[3] has stressed this possibility, pointing out that the major increase in deaths from cirrhosis has been among older women, the group recognized as being peculiarly vulnerable to chronic progressive hepatitis.

Since the peak of the epidemic in 1943, the morbidity of hepatitis has, with few exceptions, steadily declined in Denmark to 31.6 per 100,000 in 1960, the lowest annual rate ever reported. By contrast is the experience of the United States in the past decade during which we have sustained two large outbreaks; the first from 1953 to 1955, and the second be-

ginning in 1959, reaching a rate of 23.1 per 100,000 population in 1960, with a promise of an even higher rate in 1961 (Fig. 3). However, despite these relatively sharp increases in morbidity in this country, our maximum rates are greatly below those reported from other countries during periods of high incidence and actually are similar to the current rate in Denmark. Whether we actually have less hepatitis or less effective reporting is

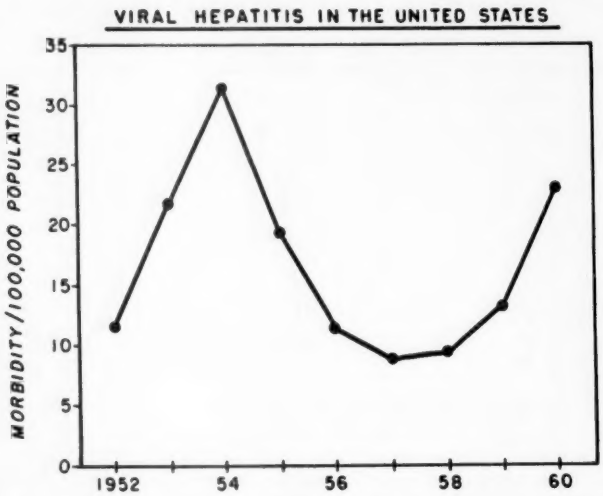

FIG. 3. The data used in preparing this chart were taken from The CDC Hepatitis Surveillance Report, Number 4, March 21, 1961.

not known, but it appears quite likely that we have more hepatitis in this country than is evident from the figures.

HEPATITIS AND VOLUNTEERS

Viral hepatitis is presently regarded by many as the infectious disease presenting the greatest number of important unsolved problems. That this may be true, however, does not reflect lack of interest, for over the past two decades, in particular, many have tried their hands at unraveling the mysteries that shroud the epidemiology, etiology, and pathogenesis of this disease. Not a few of these secrets have been elucidated, and a considerable store of information has been accumulated on the natural history of hepatitis, its caustive agents, ways of spread, clinical course, and the pathologic changes associated with it. Indispensable to the solution of many

of these problems have been human volunteers, and their roles can never be forgotten. The modern history of hepatitis is dominated by an array of studies in which they participated, beginning in 1942 and continuing interruptedly under the direction of several investigators up to the present time.* For those who have shared these experiences, it can be said that they were frequently harrowing, on occasion not without tragedy, but never without dignity. Much is owed to the relatively small number of volunteers who contributed so generously of themselves.

In retrospect, there appear to be three periods of maximal intensity of activity in the studies with volunteers. The first was during and immediately after World War II and was dominated by the pioneer work of Oliphant, Paul, Stokes and Neefe, as well as Francis, and their associates. These were exciting days, and many of the presently accepted ideas about hepatitis came into being as a result of these studies that were primarily concerned with properties of virus, routes of transmission, period of infectivity, methods of prevention of disease, and the relationship between infectious hepatitis and serum hepatitis. The second period, in the early 1950's, was largely concerned with the fruitful studies on the carrier state, particularly in serum hepatitis, by Murray and also by Stokes and Capps and their associates. The third period began in the late 1950's and is dominated by the highly productive and imaginative work of Ward and Krugman and their associates. Unfortunately, unsolved problems still loom mightily. The assiduous and often protracted attempts over the years to develop a specific serological test and to propagate human hepatitis viruses in any other medium except man were apparently unrequited,[5] and the perusal of the numerous descriptions of these investigations reveals a dismal saga of poorly rewarded ventures. Again and again over the years, several viruses, some identified and others as yet unidentified, were found in patients with hepatitis, but their relationship was never proved to be more than fortuitous. Each announcement was followed by a flurry of activity in other laboratories that soon subsided as its justification appeared impossible. Presently, however, a new wave of excitement and hope follows the claims (published and unpublished) of a number of investigators who have recently described the isolation in tissue culture of apparently new agents from patients with hepatitis.[6] One of these has been reported to produce hepatitis in volunteers after a short incubation period,[7] while another is neutralized by convalescent-phase serums from patients with serum hepatitis.[8] The outcome of these

*Specific references to all of these studies may be found in earlier reviews[3, 4] or in this paper.

promising studies is eagerly awaited. Meanwhile, new interest has been engendered in the possibility[9] that chimpanzees may acquire infectious hepatitis and transmit it to man.

STUDIES AT YALE UNIVERSITY SCHOOL OF MEDICINE

During the past two decades, many have had spasmodic and fitful interests in viral hepatitis both here and abroad, but the infrequent successes of investigations of many of the problems associated with this disease were insufficient to hold for long any but the most curiously devoted followers. However, in a few laboratories a steady and abiding interest in hepatitis has been maintained, and in them and subsequently in the laboratories of others formerly associated with them a continuity of activity and association was preserved over the years.* It is appropriate to point out that this continuance of interest was inspired largely and, indeed, supported by the Army or Armed Forces, and it is of interest to mention here that the ground work for the studies subsequently carried out in the Department of Preventive Medicine at Yale University School of Medicine under the supervision of Dr. John R. Paul was laid in Cairo in November, 1943. Following a visit from Brigadier General J. S. Simmons, Dr. Paul, then Director of the Commission on Neurotropic Virus Diseases, AEB, was ordered from Cairo to Sicily to investigate the epidemic of hepatitis that was ravaging Allied Forces. The hospitals were filled with jaundiced men, and even the corridors of the 59th Evacuation Hospital, in Palermo, were crowded with beds for patients with hepatitis. During this trip and subsequently back in the Middle East, numerous specimens of serums and feces were collected. Unfortunately, Dr. Paul was felled by hepatitis, inadvertently transmitted by the injection of human serum as a part of an experiment with sandfly fever some three months previously. It was his poor luck to be seriously sick, and it was not until early February, 1944, that the trek was made back to New Haven. The journey was not without incident; however, the race against the disappearance of dry ice and thawing of specimens was won over a tortuous route via Tunis, Rabat, Azores, and Washington. The decision had been made to enlist the aid of volunteers, and a laboratory was established in New Haven to pursue these studies. Two hepatitis viruses were recovered from the materials brought back from the Middle

*In addition to the investigators previously mentioned, it is appropriate to name the following in this country: Drs. George Mirick, Frederik Bang, Werner and Gertrude Henle, Robert McCollum, Alfred Evans, Victor Sborov, Cecil Watson, and Paul Gyorgy.

East. One of them (Virus A) came from a specimen of feces obtained from an infantryman, hospitalized outside Cairo, who had come down with hepatitis on the Anzio beachhead and produced the disease in volunteers 15 to 30 days after ingestion. The second strain of hepatitis virus (B) came from a pool of serums from eleven men in the acute phase of sandfly fever, in Cairo, and produced serum hepatitis in volunteers 60 to 90 days after parenteral inoculation. These two studies[10] initiated the work in New Haven that was carried out there, and later in additional laboratories, during the ensuing eighteen years under the sponsorship of the Commission on Neurotropic Virus Diseases and subsequently its successors, the Commission on Virus and Rickettsial Diseases and the Commission on Viral Infections, and, the Commission on Liver Disease.

Following the cessation of war in Germany and during the early years of the Occupation, the high incidence of hepatitis in our troops commanded continuing interest in this disease. A center for the care of patients with hepatitis was established by the Army in 1947 at the 120th Station Hospital at Bayreuth, in an institution formerly used by the German Luftwaffe; in 1949 it was transferred to the 98th General Hospital in Munich. Dr. Paul and members of his group functioned in a consultant capacity and furnished certain civilian personnel.* During this period, numerous studies of an epidemiologic and clinical nature were made in addition to continued unsuccessful attempts to transmit hepatitis viruses to laboratory animals and tissue culture. For years, these centers were vital sources of material that was sent back to this country for use in virologic and serologic investigations. The frequent trips to Germany for purposes of consultation furnished an unusual opportunity to observe the medical experiences of United States troops against the background of the breath-taking, socio-economic changes that took place in postwar Germany. As the number of our Occupation troops diminished, and as the incidence of hepatitis subsided, the need for a center was no longer apparent; in 1953, the center at Munich was closed and patients with hepatitis were sent to the Army Hospital at Landstuhl. However, despite the lessened frequency of disease, the Armed Forces in Germany and elsewhere have continued to provide, right up to the

*The Hepatitis Research Team was formed here in 1947 and during the next three years the following men acted as Clinical Directors: Drs. Horace T. Gardner, 1947-1948; William E. Swift, Jr., 1948-1949; and James W. Colbert, 1949-1950. Dr. Dorothy M. Horstmann acted as Director of Virus Laboratory, 1949-1950. During 1950-1952, the clinical investigations were largely the work of the Medical Staff of the 98th General Hospital, and important in this group were Col. Robert S. Nelson, MC, Lt. Col. Helmuth Sprinz, MC, and Capt. Alfred S. Evans, MC.

present, a reason for numerous studies by members of the group associated directly or indirectly with the laboratory at Yale University. In addition, members of this group carried out a variety of studies on this disease both here and abroad, including Japan, Korea, North Africa, Turkey, India, and Italy.

FACTS AND FANCIES ABOUT HEPATITIS

It is not unexpected that during two decades of intensive work several changes in thinking about hepatitis should occur; at times these changes were clear-cut, but often it is not easy to mark the time or place where one concept merged with another. For example, early studies[11, 19] appeared to show that the administration of normal human gamma globulin prevented epidemic hepatitis when the diagnosis was based on the presence of jaundice. However, recent observations,[18] based on more subtle tests of hepatic function, have indicated that the effect is modifying rather than preventive since the incidence of anicteric hepatitis is not significantly changed by the administration of gamma globulin. In addition, the earlier observations suggested that as little as 0.01 ml./pound was effective, while the recent results indicate that although this may be effective in children, it is not sufficient in adults.

In regard to treatment, the pendulum has swung far as manifested by reports of duration of hospitalization in certain groups of patients. In 1943-1944 in Egypt, the average period of hospitalization was thirty days. Following the concern that developed in North Africa and Sicily over relapse in patients rested for short periods, the duration of hospitalization increased sharply to 50 days in the Mediterranean Theatre in 1943-1945, 60 days in Germany in 1947-1949, and 89 days in Japan in 1950-1951. This increase in loss of time was prompted largely by fear of the development of relapse or chronic hepatitis and lack of knowledge of the factors that potentiated them rather than by any recognizable increase in severity of disease. The overconcern of medical officers and civilian consultants that developed was attested by the incredulity that greeted a suggestion made in 1950,[14] based on observations at the Hepatitis Center in Bayreuth, that patients with hepatitis could start increased activity and return to duty sooner than hitherto expected. That this seemingly "uncontrolled" observation was true, however, was borne out by the results of subsequent studies conducted in Japan by Chalmers *et al.*,[15] indicating that a somewhat less stringent program of rest was not only possible but actually desirable. Even more difficult to overcome was the "inherited" misapprehension that fats are poorly tolerated by patients

with hepatitis. In spite of carefully controlled studies made years ago[18] showing the desirability of dietary fats, particularly of dairy origin, there apparently persists in the minds of many physicians almost a reflex aversion to their use.

Early in World War II, it was not readily accepted by some that hepatitis without jaundice ever occurred, and there were those who disparaged the idea and spoke scornfully of "chemical hepatitis." How far around the wheel has turned is now attested by the generally accepted belief that anicteric hepatitis, especially among children, is far more common than hepatitis with jaundice. Further exploration of this concept, particularly in the light of recent studies by Ward and Krugman[17] now justifies the question whether the disease called hepatitis is actually primarily a liver disease or, possibly, a generalized systemic disease in which the liver need not always be involved.

Despite the fact that at least two strains of virus are known to cause hepatitis, our concept of the variations of the disease they may produce is rather limited and it is actually not known whether infection may not occur, and possibly far more frequently without involvement of the liver. Epidemic hepatitis is an acute enteric infection with generalized systemic manifestations. Although the most striking and apparent effect of hepatitis virus A in man is on the parenchymal cells of the liver, too little is known about this virus to allow conclusive definition of the pathogenesis of the disease it causes. The preponderance of gastrointestinal complaints, as well as the roentgenographic and gastroscopic changes, the pathologic findings at necropsy, the demonstration of virus in the feces during the early days of illness and its transmission by feeding, directed attention years ago to an alimentary phase of disease. Interest in the importance of this phase and its relationship to multiplication and excretion of virus prompted a single experiment in the laboratory in New Haven as long ago as 1945[18] when feces collected from experimentally infected volunteers fifteen days before the onset of their disease were fed to three other volunteers. None of these three men acquired hepatitis, and the period of infectivity of the feces during the incubation period remained unknown until 1958 when Ward and Krugman[17] again called attention to the importance of the alimentary phase of disease on the basis of results of their experiments that demonstrated the presence of virus in the blood and feces of children 25 days after they had been fed virus and two to three weeks before the appearance of jaundice at a time when there were neither clinical manifestations nor abnormalities of hepatic function. These observations were in keeping with the epidemiologic

evidence that for years had suggested the importance of the asymptomatic fecal carrier in the transmission of disease. Since virus apparently may multiply in the gastrointestinal tract in this unobtrusive fashion without any measurable impact on subjects who eventually come down with clinical evidence of hepatitis, there is good reason to suspect that the initial alimentary phase may, in a certain percentage of people, constitute the whole expression of disease. That viremia was also present at this time is of particular interest in relation to the subsequent demonstration of viremia in another subject by Ward and Krugman[17] thirty-seven days after the ingestion of virus at a time when he was asymptomatic but was believed to have acquired epidemic hepatitis on the basis of a modest increase in the serum transaminase as the only indication of hepatic involvement. Since viremia occurs during the asymptomatic and biochemically normal alimentary phase of the incubation period of a disease that eventually may culminate in jaundice and since viremia also may be present at a time appropriate for the onset of disease but actually without any evidence of it except for a modest increase in serum transaminase, it does not require any soaring of the imagination to conceive that a certain percentage of persons who ingest hepatitis virus A may have only an alimentary infection and viremia but without recognizable hepatic disease. That this could be more common in extreme youth might be suggested by the apparent frequency of hepatitis without jaundice in young children. Proof of the existence of such a broad spectrum in type and severity of infection awaits the development of a specific serologic test to measure the immunologic experience in man.

The recently acquired information on the potential infectivity of patients in the incubation period of epidemic hepatitis takes its place in an interesting manner with what has been known for a long time about serum hepatitis. Between 1944 and 1946, a series of observations in Philadelphia and New Haven showed that strains of serum hepatitis virus could be recovered from the blood of experimentally infected volunteers 87, 60, and 16 days before the onset of illness, when they had no evidence of clinical disease nor abnormalities of hepatic function.[19-21] The factors that potentiate this curious prolonged equilibration between virus and host in serum hepatitis and the factors that break it, making clinical disease possible, are unknown. However, it may safely be presumed that on many occasions no break in equilibration occurs and that viremia persists for prolonged periods without any evidence of illness. The importance of the asymptomatic carrier as the repository and medium for transfer of serum hepatitis virus is generally appreciated.

Recognition of the alimentary phase with viremia in epidemic hepatitis and the prolonged period of viremia in serum hepatitis without any evidence of hepatic dysfunction has raised the question whether the action of virus itself produces hepatic damage if and when it occurs. Despite the fact that descriptions of varying sized bodies visualized by electron microscope in the liver cells of patients with acute hepatitis have appeared, there is no conclusive evidence, as yet, that these represent hepatitis virus. Indeed, the liver may be a poor place to seek hepatitis virus, particularly after the onset of clinical disease when biopsies are ordinarily made. The possibility has been suggested, and might be considered particularly in serum hepatitis, that the hepatic injury which potentiates the appearance of jaundice is not only the result of direct action of virus but also the result of the interaction of virus or some product of tissue necrosis and the antibody evoked by it.[23] While there is no proof positive for this speculation, reflection on certain hyperergic manifestations of serum hepatitis, such as the occurrence of itching, hives, arthralgia, and, on occasion, maculopapular rash on the legs before jaundice, might prompt the suspicion that the hepatic injury is a similar manifestation. The apparent rarity of the classical histologic changes of adult viral hepatitis in infancy and the fact that jaundice is uncommon in hepatitis in early childhood but increases with age support the possibility that maturation may be important in providing the conditions that result in its appearance. In recent years, there has been a resurgence of interest in the possibility that the development of chronic hepatitis is effected by auto-antibodies. The serologic evidence that might be adduced to support this concept has been recently reviewed[28] and it is indeed true that chronic hepatic disease may flourish in a biologic environment containing auto-antibodies. Popper *et al.* have demonstrated the presence of gamma globulin by fluorescence microscopy in the livers of patients with chronic hepatitis, and more recently they have described both nuclear and cytoplasmic binding substances in the serums of patients with acute and chronic hepatic disease.[24] As yet, the real significance of all of these findings is not defined, and it is impossible to say that auto-antibodies have a causative role in the production of hepatic disease. Further investigations may clarify this situation.

Among the numerous unsolved problems in viral hepatitis is the relationship between the epidemic disease and serum hepatitis. It is generally but not universally believed that the viruses causing them are different and the differences between them are familiar to all. Experimentally, Virus A has been found in the feces and blood and can be trans-

mitted by feeding or parenteral inoculation, producing disease with a short incubation period. In contrast, Virus B has been found only in the blood and apparently can be transmitted only by parenteral inoculation. Contact cases are common in the epidemic disease and rare in serum hepatitis. Lastly, there is no cross immunity experimentally between Virus A and Virus B, and in the field it was shown that men who had had serum hepatitis were apparently even more susceptible to epidemic hepatitis. It seems impossible not to admit that real differences exist between these two viruses, yet it is of interest to speculate on how they come about. Are they basically different viruses or does Virus A represent a master strain from which a number of strains of Virus B have developed? Among the imponderabilities of Virus B is the concept that it depends on the needle for survival. Despite the frequency of chance for infection by this route, it would appear to be a precarious way of existence unless there were some continuing source of supply of virus either by other ways of transmission or emergence. Years ago in New Haven, it was shown that Virus A inoculated parenterally was excreted in the feces[25] — this has not been shown for Virus B; however, the rare occurrence of hepatitis in wives of soldiers with serum hepatitis and the epidemic of serum hepatitis described by Mirick *et al.*[26] in which contact cases appear to have occurred suggest that this matter is not completely settled. Of particular interest in this situation is the possibility that the survival of Virus B actually depends not on the needle but on the continuing emergence of new strains in man so that there is always available a new source of supply. The more recently available information on the duration of viremia in epidemic hepatitis makes it quite clear that infection with Virus A may occur with little or no sign of hepatic disease. It had been thought earlier, on the basis of experiments with volunteers, that in those patients who had classical epidemic hepatitis with jaundice, the presence of virus in the feces and blood was a limited experience, terminating early in the acute phase of disease.[2] At all events, attempts to recover virus from the feces and the blood four weeks, three months, and one year after onset of disease were unsuccessful. Subsequent studies, however, showed that Virus A might remain in the feces as long as 5 to 16 months after the onset of disease.[27] It is entirely possible, and indeed likely, that viremia also may persist in some patients, particularly in those who have essentially an alimentary disease for prolonged periods. Under such conditions, it is worthy of consideration that Virus A might eventually undergo alterations that would condition a new behavior, emerging with differences that are now regarded as characteristic of

Virus B. Under certain circumstances, some previously held properties, such as capacity for excretion in the feces after parenteral inoculation, might be maintained in a limited fashion and account for the occasional occurrence of apparent contact cases in serum hepatitis. In this way, an ever-present source would be available for the evolution of strains of Virus B from Virus A, guaranteeing its biologic survival, although its transmission might be almost completely dependent on the needle.

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SAUL KRUGMAN*
ROBERT WARD**

Department of Pediatrics, New York University School of Medicine and Department of Pediatrics, University of Southern California, Los Angeles

INFECTIOUS HEPATITIS: CURRENT STATUS OF PREVENTION WITH GAMMA GLOBULIN

INTRODUCTION

The national incidence of 28,635 cases of viral hepatitis† during the first four months of 1961 was the highest for any comparable period since the disease became reportable in 1952.[1] The attack rate was 16 per 100,000, a figure more than double the 6.9 per 100,000 rate reported for the first 16 weeks in 1960.

The age distribution curves of infectious hepatitis showed a striking shift toward the adult age groups in 1959 and 1960. In past years the highest incidence was observed in the pediatric age group, i.e., persons under 15 years of age.[2] Currently available statistics for the New England and Middle Atlantic States indicate that more than 50 per cent of the reported cases of hepatitis were adults over 20 years of age.[1]

The increasing incidence of infectious hepatitis, especially in adults, has been a source of concern to physicians and public health officials. This situation has created a renewed interest in gamma globulin, the only available prophylactic agent against this disease. The increased demand for immune serum globulin has highlighted the importance of defining its virtues and limitations as well as resolving the current confusion in regard to optimum dosage.

THE GAMMA GLOBULIN DILEMMA

An adequate evaluation of the efficacy of gamma globulin in the prevention of infectious hepatitis requires precise information about i) the susceptibility of the host, ii) the type of exposure to the virus, and iii) the IH antibody content of the preparation used.

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*Professor of Pediatrics, New York University, School of Medicine.

**Professor of Pediatrics, University of Southern California. Formerly Research Assistant (Assistant Professor) in Section of Preventive Medicine, Yale University School of Medicine, 1943-1945.

†This includes both infectious hepatitis (IH) and serum hepatitis (SH). The number of SH cases, however, represents only a very small fraction of the total figure.

Host Susceptibility. The susceptibility of the host cannot be measured accurately. At the present time a serological test is not available for the titration of infectious hepatitis neutralizing antibodies. In general children are more susceptible than adults. Most adults have acquired their immunity following an unrecognized anicteric infection contracted in childhood.

The incidence of infectious hepatitis among children and adults following intimate household contacts was reported by Brooks *et al.*[3] and Hsia *et al.*[4] As indicated in Table 1 the attack rate in children was 32

TABLE 1. INCIDENCE OF INFECTIOUS HEPATITIS IN CHILDREN AND ADULTS FOLLOWING INTIMATE HOUSEHOLD CONTACT

	Controls who did not receive gamma globulin					
	*Study No. 1**		*Study No. 2***		*Combined totals*	
Study groups	*No. exposed*	*No. with hepatitis*	*No. exposed*	*No. with hepatitis*	*No. exposed*	*No. with hepatitis*
Children < 15 years	15	18 (35%)	40	11 (27%)	91	29 (32%)
Adults > 15 years	63	0 (0%)	55	2 (4%)	118	2 (1.7%)

* From, Brooks, Hsia and Gellis, *New Engl. J. Med.*, 1953, *249*, 58.
** From, Hsia, Lonsway and Gellis, *New Engl. J. Med.*, 1954, *250*, 417.

per cent; in 118 adults over 15 years it was only 1.7 per cent. The adults in these studies were subjected to the same exposure which was followed by an attack rate of 32 per cent in children. Consequently, the resistance to infection was due most likely to immunity rather than inadequate exposure. An inoculation of a minimal dose of gamma globulin or even physiological saline could have been erroneously credited with reducing the incidence of infectious hepatitis to a mere 1.7 per cent of 118 exposed adults.

Unpublished observations at Willowbrook State School[5] have indicated that resistance to infection is related to the socio-economic status of the patient. Negro children who have lived under conditions of crowding and poor sanitation are more immune than white children of comparable age from more highly sanitary areas.

In contrast to measles, infectious hepatitis in children occurs for the most part as an unrecognized infection. Consequently, a history of previous

infection is usually not available. At the present time, host susceptibility is crudely related to the age and socio-economic status of the person.

Type of Exposure. The virus of infectious hepatitis is excreted in the stools and is present in the blood of patients in the preclinical as well as the acute phase of the disease. Consequently, the virus is disseminated via the intestinal-oral circuit through human association or by the parenteral administration of infected blood or blood products. The attack rate of infectious hepatitis is directly proportional to the intimacy and duration of exposure. The determination of an adequate exposure is difficult because of the lack of a laboratory tool to identify the virus in the suspected patient or in the individual with unsuspected subclinical infection.

Antibody Content of Gamma Globulin. The IH neutralizing antibody content of gamma globulin cannot be measured. Since gamma globulin is prepared from large pools of adult plasma, the antibody content will be determined by the immune status of the donor group. The high incidence of infectious hepatitis in adults during 1959 and 1960 is a reflection of a non-immune population. Gamma globulin prepared from adult plasma pools collected at this time may have a low antibody content. It is conceivable that a dose based on 0.01 ml./lb. of patient weight, using gamma globulin prepared from American Red Cross Blood collected in 1940, may provide more IH antibody than a much larger dose of 1959-60 preparation. The variability of the IH antibody content of gamma globulin probably parallels the variability of poliovirus antibody.

In summary, the physician who attempts to evaluate the efficacy of gamma globulin in the prevention of infectious hepatitis is confronted by the triple dilemma of lack of precise knowledge concerning i) the susceptibility of the host, ii) the adequacy of exposure to infective virus, and iii) the antibody content of gamma globulin. In view of these difficulties, recommendations for a particular dose of gamma globulin should be based on controlled studies in comparable age groups of similar socio-economic status with comparable exposure and comparable lots of gamma globulin.

RECOMMENDED DOSE OF GAMMA GLOBULIN

The dose of gamma globulin currently recommended for the prevention of hepatitis with jaundice ranges between 0.01 ml. and 0.06 ml. per pound of body weight administered promptly after exposure. The 1960 edition of the American Public Health Association Control of Communicable Diseases in Man recommends "immune serum globulin, 0.01 ml. per pound of body weight intramuscularly. . . ."[8] Enclosures in vials

of gamma globulin distributed by the American Red Cross suggest a dosage of 0.02 ml. per pound of body weight. The Armed Forces tri-service medical technical bulletin on "Immunization"* recommends a dosage of 0.05 ml. per pound for military personnel. The American Academy of Pediatrics Committee on the Control of Infectious Diseases in its 1961 report states "the recommended dose in children is 0.04 ml./kg. body weight (approximately 0.02 ml. per pound) ; adults may require up to 0.1 ml./kg.[7] (approximately 0.05 ml. per pound)." On the basis of controlled institutional studies completed in 1960, Krugman *et al.*[8] suggested that the optimum dose of gamma globulin for children and especially adults is 0.06 ml. per pound of body weight. More recently[9] Gellis and McComb recommended the smaller 0.01 ml. per pound of body weight dose for both children and adults.

The following summary of published and unpublished controlled trials of gamma globulin is reviewed in an attempt to clarify some of the confusion in regard to dosage for children and adults.

STUDIES WITH LARGE DOSES OF GAMMA GLOBULIN (0.06 ml.-0.15 ml./lb.)

The first reported trial of gamma globulin by Stokes and Neefe[10] in 1944 was conducted in a children's camp during an explosive waterborne epidemic of infectious hepatitis. Gamma globulin was administered in a dose of 0.15 ml. per pound of body weight. The attack rate was 45 per cent in the control group as compared with 5.7 per cent in the gamma globulin group. Later in 1944, these observations were confirmed by Havens and Paul[11] who studied an epidemic of infectious hepatitis in an orphanage in New Haven. Following a dose of 0.06 ml. to 0.12 ml. gamma globulin per pound by body weight hepatitis with jaundice was observed in 23 per cent of the control group and 2 per cent of the inoculated group. Both studies demonstrated about a 90 per cent reduction in incidence of icteric hepatitis following large doses of gamma globulin administered during acute explosive epidemics of the disease in children.

The first reported study in adults was conducted in the Armed Forces by Gellis and associates.[12] The attack rate of infectious hepatitis among 683 controls was 3.7 per cent as compared with 1.0 per cent of 406 soldiers inoculated with gamma globulin 0.06 ml. per pound of body weight. Thus, this dose of gamma globulin in adults was 73 per cent effective in contrast to the 90 per cent effectiveness in children. The

*Army TB MED 114, Navy MED P-5052-15 and Air Force Pamphlet-5-1, dated 22 December, 1959.

effectiveness in our Willowbrook trials with gamma globulin 0.06 ml. per pound of body weight also exceeded 90 per cent in a controlled group of patients, predominantly children.[8]

STUDIES WITH SMALL DOSES OF GAMMA GLOBULIN (0.01 ml. per lb.)

The first trial with small doses of gamma globulin (0.01 ml./lb.) was conducted by Stokes and associates[18] in a training school for children during an epidemic of infectious hepatitis. The attack rate in the control group of children was 54 per cent as compared with 2 per cent in the inoculated group. The effectiveness which exceeded 90 per cent was comparable to the results achieved with the larger doses. Cases of hepatitis continued to occur in the control group for at least 8 months after the trial began. After the first month, hepatitis with jaundice was not detested in the gamma globulin group. This prolonged protective effect was postulated by Stokes to be due to passive-active immunity.

Hsia, Lonsway and Gellis[4] confirmed Stokes' observations in their well controlled study of household contacts. Alternate members of an exposed family received gamma globulin in a dose of 0.01 ml. per pound of body weight. The attack rate of icteric hepatitis was 27 per cent of 40 controls and 2 per cent of 57 inoculated children. Subsequently, the studies by Drake and Ming[14] provided additional confirmation of the efficacy of the small doses of gamma globulin.

In contrast with previous reports, our Willowbrook studies[8, 15] with the 0.01 ml. per pound body weight dose of gamma globulin showed the following significant differences: i) an attack rate of 2 per cent in 2988 controls as compared with 0.7 per cent of 1224 inoculated inmates, that is a 54 per cent effectiveness, and ii) no protection among 125 adult institutional employees as compared with 456 controls; an attack rate of 3.2 per cent in the gamma globulin group and 1.7 per cent in the controls. In an attempt to clarify this discrepancy, the old trials of gamma globulin were re-evaluated and new ones were begun.

RE-EVALUATION OF WILLOWBROOK TRIAL WITH GAMMA GLOBULIN 0.01 ml. lb.

The failure of the small dose of gamma globulin to protect adequately against hepatitis in Willowbrook may have been due to at least three factors: i) the possibility of a low hepatitis antibody content of gamma globulin; ii) the age factor and iii) the peculiar endemic situation in the institution.

Antibody Content of Gamma Globulin. A single lot of gamma globulin* was employed in the Willowbrook trial. The protective effect of this material would be decreased if it were lacking in hepatitis antibody. Unfortunately, a serological test to measure infectious hepatitis antibody is not available at this time. However, a suggestion of the presence or absence of hepatitis antibody may be obtained by measuring the measles and poliomyelitis neutralizing antibody content of gamma globulin.

An aliquot of the gamma globulin used for the Willowbrook trial was tested** for the presence of neutralizing antibody. The neutralizing antibody titers were as follows: measles — 1:200; poliomyelitis, type 1 — 1:256, type 2 — 1:512 and type 3 — 1:104. These results provided indirect evidence that the gamma globulin employed in this study was probably adequate and low potency was not likely to be the responsible factor.

The Age Factor. It is well recognized that infectious hepatitis in adolescents and adults is apt to be a more severe, more protracted and more debilitating disease than in younger age groups. Inapparent and mild infections are much more common in children. The dividing line in this particular host response appears to be puberty. It seems reasonable to postulate that the same small dose of gamma globulin which will protect against the insignificant childhood disease may not be as effective against the more formidable adult disease.

The above hypothesis would explain the failure of a small dose of gamma globulin to protect the adult attendants. It would also explain the decreased efficacy of this dose which gave only 54 per cent protection for the inmates. An analysis of the age distribution of 4177 inmates who participated in the study revealed that 42 per cent were over 15 years of age.[15]

The Peculiar Endemic Situation in the Institution. Infectious hepatitis has been endemic in Willowbrook for the past eight years. The attack rate of the icteric disease has ranged between 1 and 3 per cent per annum. Susceptible contacts have been subjected to a continuous, low-grade, smouldering type of exposure. In contrast, in other institutional studies[10, 11, 13, 14] the attack rate of hepatitis with jaundice in children has ranged between 21 and 45 per cent. These epidemics have been explosive and short term in type, ranging between one and eight months. In the studies of household contacts by Brooks *et al.*[3] and by Hsia *et al.*[4] the

* Courtland Laboratories, gamma globulin lot no. 5009.

** We are indebted to Dr. Maurice Hilleman, Director, Merck Institute of Therapeutic Research for performing the neutralizing antibody titrations.

attack rate in children was similar to the institutional epidemics ranging between 27 and 35 per cent.

The decreased efficacy of the 0.01 ml. per pound dose of gamma globulin in the Willowbrook studies may be related to the peculiar endemic situation in the institution. Under these conditions of continuous, long term exposure the small dose of gamma globulin may be sub-optimal.

EFFECT OF DOSAGE OF GAMMA GLOBULIN ON PASSIVE ACTIVE IMMUNITY

Stokes and his co-workers[18] were the first to demonstrate prolonged protection against icteric hepatitis following administration of gamma globulin to persons exposed in institutional outbreaks. These authors postulated that the gamma globulin markedly attenuated the disease in the exposed persons, thereby inducing a subclincal infection. Subsequently, our studies[8, 15] tended to confirm this hypothesis.

It has been suggested that a large dose of gamma globulin will be followed by too much passive immunity and not enough active immunity. Preliminary studies reported by our group last year[8] did not support this hypothesis. Recently, we have had an opportunity to extend these observations at the Willowbrook State School.*

Since May 1959, 85 patients have been studied intensively for a six month period following admission to this institution in which infectious hepatitis is endemic. A total of 40 patients received gamma globulin, 0.06 ml. per pound on admission; the remaining 45 patients were uninoculated controls.

Each patient was examined and bled at least once weekly, and more often if necessary. The following biochemical tests of liver function were performed: serum transaminase (SGOT), thymol turbidity, and cephalin flocculation. Any suggestion of skin or scleral icterus was confirmed by a serum bilirubin determination. The following criteria were established for a diagnosis of hepatitis *with* jaundice: evidence of clinical icterus confirmed by a serum bilirubin more than 1.0 mg. per 100 ml. and an abnormal SGOT activity (more than 60 units). Criteria for a diagnosis of *anicteric* hepatitis included a crescendo-like rise in SGOT activity plus an abnormal elevated thymol turbidity.

The results of this study are shown in Figure 1. It is apparent that gamma globulin in a dose of 0.06 ml. per pound did not prevent hepatitis infection. However it did delay the occurrence of jaundice until the fifth

* We are indebted to Dr. Paul H. Hoch, New York State Commissioner of Mental Hygiene and to Dr. Harold H. Berman, Director of Willowbrook State School for their cooperation.

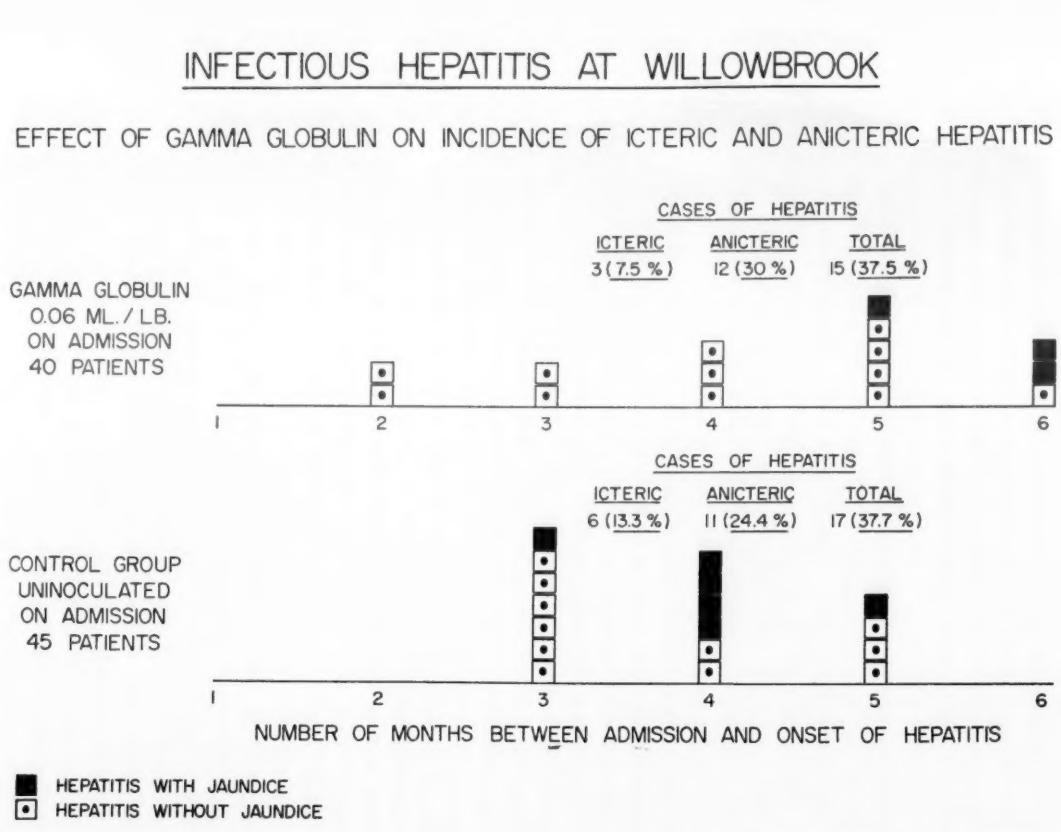

FIG. 1. Effect of gamma globulin, 0.06 ml. per pound of body weight on the incidence of icteric and anicteric hepatitis.

and sixth month after admission to the institution. In the control group, hepatitis with jaundice appeared earlier and in larger numbers.

As indicated in Figure 1 the attack rate of hepatitis with jaundice was 7.5 per cent for the gamma globulin group as compared with 13.3 per cent for the control group. The overall attack rate for combined icteric and anicteric-hepatitis was almost identical for both groups, 37.5 per cent and 37.7 per cent respectively.

The gamma globulin* employed for these studies was tested for measles and poliomyelitis neutralizing antibody. The results of the titration were as follows: lot 234-2: measles—1:400, poliomyelitis type 1—1:416, type 2—1:630 and type 3—1:208 and lot 310: measles—1:200, poliomyelitis type 1—1:630, type 2—1:630 and type 3—1:256. These data provided indirect evidence of the presence of infectious hepatitis antibody in the gamma globulin.

In summary, under the conditions of this study the larger dose of gamma globulin (0.06 ml. per pound) did not prevent hepatitis infection. Consequently it probably did not interfere with passive-active immunity. Administration of the larger dose was associated with a significantly lower incidence of hepatitis with jaundice. These observations confirmed previously reported studies[8] which indicated that the protective effect of a 0.06 ml. per pound dose persisted for at least five months.

CONCLUSIONS

The available evidence based on published controlled studies will not support a "blanket" recommendation for a 0.01 ml. per pound dose of gamma globulin for all age groups and for all types of exposure to infectious hepatitis. In view of the probable variability of the IH antibody content of gamma globulin it is suggested that the current minimum dose be 0.02 ml. per pound of body weight. The maximum dose need not exceeed 0.06 ml. per pound.

The minimum 0.02 ml. per pound dose is recommended for children who have had a transient, short term type of exposure; for example, a household or school contact, an explosive institutional epidemic. The efficacy of this dose for susceptible adults is unknown.

The larger 0.06 ml. per pound dose is recommended under the following circumstances:

*Gamma globulin prepared by E. R. Squibb & Sons, New York from plasma furnished by the American Red Cross, lot numbers 234-2 and 310.

1) for children and adults subjected to prolonged, continuous exposure in a highly endemic area;
2) for pregnant and postmenopausal famales;
3) for patients with pre-existing hepatic disease.

A universal recommendation of 0.01 ml. per pound of body weight may not prevent hepatitis with jaundice in a significant number of exposed *susceptible* adults. On the other hand, administration of large doses of gamma globulin to many adults with a high index of immunity will waste an expensive product in limited supply. Under these circumstances a public health officer may be justified in releasing the minimal dose. However, it should be recognized that the most *feasible* dose from the public health viewpoint is not always the most *optimum* dose from the individual viewpoint.

ADDENDUM

From 1, February 1960 until 1, November 1961, one thousand new employees were appointed at the Willowbrook State School. Of 399 adults who received 4 ml. of gamma globulin* intramuscularly (approximatly 0.03 ml./lb. body weight) two (0.5%) contracted hepatitis with jaundice. Of 601 uninoculated adults, 27 (4.5%) acquired the disease. Under the conditions of this study this dose of gamma globulin was 90% effective in reducing the incidence of hepatitis with jaundice.

ACKNOWLEDGMENT

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*E. R. Squibb lot number 234-1.

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HEATHER DONALD MAYOR*
JOSEPH L. MELNICK**

Department of Virology and Epidemiology, Baylor University College of Medicine Houston, Texas

INTRACELLULAR AND EXTRACELLULAR REACTIONS OF VIRUSES WITH VITAL DYES†

Vital dyes, long used in cytologic studies of living cells, are now assuming increasingly important and diverse roles in virus research. They are employed as visual indicators of infection in virus assays, and their involvement in the photo-inactiviation of viruses is well established. In particular, studies with such dyes as neutral red, toluidine blue, proflavin, and acridine orange have been most fruitful. The fluorochrome, acridine orange, is of special importance because it has the dual capacity to act as a vital stain and as a cytochemical reagent for the detection of nucleic acids. Moreover, under conditions of controlled pH and concentration, acridine orange can differentiate, at the microscopic level, between RNA, double-stranded DNA, and single-stranded DNA.

In this paper, we have brought together several aspects of our recent work related to the use of vital dyes in virus research.[1-8] Included are studies on: the photo-inactivation enhancing effect of dyes incorporated into developing poliovirus; the multiplication of poliovirus using correlated cytochemical and fluorescent antibody techniques; and the use of acridine orange as a differential reagent for nucleic acids in purified viruses.

THE INCORPORATION OF NEUTRAL RED AND ACRIDINE ORANGE INTO DEVELOPING POLIOVIRUS AND THE PHOTOSENSITIVITY OF VIRUS PRODUCED BY STAINED CELLS

It has been observed in several laboratories that virus plaques may be reduced in size and number in the presence of the vital dye, neutral red. Because inhibition apparently occurs somewhat irregularly, experiments were designed to determine what added influence light might have on the growth of poliovirus in cells containing neutral red. The combined action of light and acridine orange was also tested in a similar fashion.

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*Professor of Virology and Epidemiology. Formerly, Fellow (National Research Council), and subsequently Professor of Epidemiology, Department of Preventive Medicine, Yale University School of Medicine 1941-1957.

**Assistant Professor of Virology.

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MATERIALS AND METHODS

Tube cultures of monkey kidney cells were grown in Hanks' medium plus 2% calf serum and 0.5% lactalbumin hydrolysate, until cell sheets, representing about 10^5 cells, were formed. Melnick-Earle's medium, was used for maintenance. Virulent (Mahoney) and attenuated (LSc) strains of poliovirus were compared in the test system. Each tube was inoculated with 1 ml. containing $10^{5.5}$ PFU of virus. After adsorption for one hour, the cultures were washed with warm medium and 1 ml. of maintenance medium plus dye was added.

Dyes. Either neutral red or acridine orange was incorporated into the maintenance medium at concentration levels ranging from $10^{-5.1}$ to $10^{-5.4}$ About two hours were allowed for dye to be taken in by the cells before any light was applied.

Light exposure. An effort was made to prevent undue exposure to light of the developing (or harvested) virus. Necessary manipulations were carried out in subdued lighting and culture racks were enveloped in aluminum foil to prevent extraneous light from reaching the cells. Cultures were kept in continuous contact with dye, in the dark, for a period of eight hours, insuring that virus development would take place in stained cells. Some of the tubes were carried in darkness for the entire eight hours; others, taken at different periods, were exposed to light for one hour at 37°C. For this purpose, three 20 watt "daylight" type fluorescent lighting tubes were placed eight to nine inches away from the exposed cultures. Following this, the cultures were returned to the dark for the remainder of the eight-hour test period. At the end of eight hours, all tubes were harvested and tested for infectious virus. For control, "normal" mature virus (i.e. produced in the absence of any dye) was incubated with dye in the dark and then exposed to light for one hour before testing for infectivity.

Harvest and assay. At harvest, each tube was subjected to two cycles of freezing and thawing. After the second thawing the harvests from five tubes collected at each time interval were combined, centrifuged to remove cell debris, and the supernates titrated by the plaque technique. Details of the procedure have been described.[8]

RESULTS

In the presence of either neutral red or acridine orange, poliovirus production was inhibited to some extent, even in the dark. With the addition of light, however, inhibition was markedly increased (Table 1). This effect was easily demonstrable with either dye, neutral red or acridine orange, and no differences were noted in tests with virulent and attenuated Type 1 strains of virus. The time at which light was applied was not a factor in the inactivating process and it was found that light enhancement occurred after harvest as well as during the virus developmental period, provided that the harvested virus had been grown in the presence of dye. Light had no effect on virus produced in the absence of dye even when the latter was added before exposure to light.

DISCUSSION

The reduction in infective virus was due to the effects of light on virus developed in the presence of neutral red, since the dye and light had no effect on the mature virus grown in the absence of neutral red. Exposure to light resulted in a marked reduction in infectivity of virus which had been developed in the dark in cells containing neutral red or acridine orange. The possibility that the effect was related to alterations produced in the cells seems remote, in view of other evidence. Thus, it appears most likely that neutral red becomes incorporated into poliovirus developing in the presence of the dye.

TABLE 1. EFFECT OF LIGHT ON VIRUS GROWN IN PRESENCE OF NEUTRAL RED IN THE DARK

	PFU titers after incubation in dark 1 hour		*PFU titers after incubation in light 1 hour*		
Neutral red concen.	*Av. 3 exp.*	*Log_{10} inhibition in dark*	*Av. 3 exp.*	*Log_{10} inhibition in light*	*Log_{10} inhibition light-dark**
Mahoney:					
0	5.75	0	5.73	0	0
1 : 240,000	4.4	1.35	<1	>4.73	>3.38
LSc:					
0	5.1	0	5.1	0	0
1 : 120,000	3.7	1.4	1.75	3.35	1.95

* Inhibition due to light over and above that due to neutral red alone plus one hour incubation at 37° C. in the dark.

Our experiments, when compared with previous work of other investigators, indicates a similarity between the biological effects of neutral red and of other photodynamically active dyes such as proflavin, toluidine blue, and acridine orange. This is not surprising when one considers the formulae of these compounds (Figure 1). Proflavin is an acridine and has the formula shown with a carbon in the X-position. Neutral red, a quinonimide dye, has a nitrogen in the X-position. Toluidine blue is yet another example of a quinonimide dye. The similarities between the formulae are obvious. Tricyclic photodynamically active dyes have the general formula shown at the bottom of Figure 1, "X" indicating carbon or nitrogen and "Y" indicating sulphur, oxygen, or nitrogen. All are basic heterotricyclic compounds with an affinity for nucleic acid.

Hiatt and his coworkers[4,5] have found that enteroviruses cannot be inactivated by toluidine blue and white light, whereas vaccinia and adenoviruses are readily inactivated under the same conditions. The susceptibility of phage to the action of an active dye and light appears from their work to be linked to the permeability of the virus, since there is a correlation between these experiments and osmotic permeability of the phage as measured by osmotic disruption experiments.

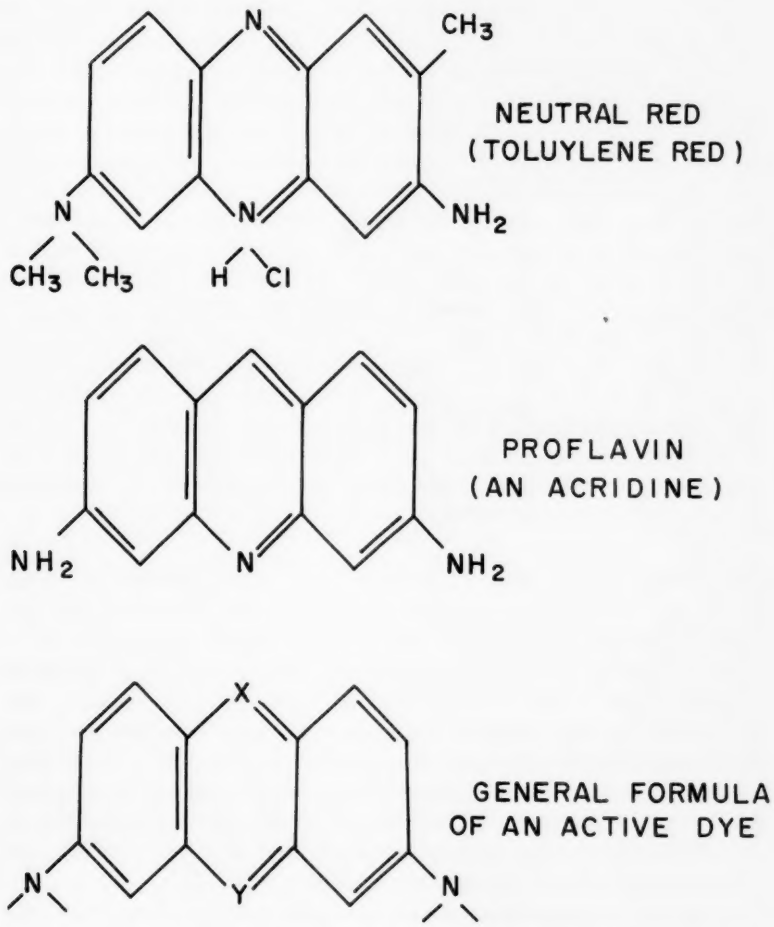

FIG. 1. Structural formulae of photodynamically active dyes.

Schaffer[8] has recently found that proflavin cannot attach to mature poliovirus particles, whereas it can be incorporated into developing poliovirus. In the course of our study, a similar sort of phenomenon was found to occur with neutral red: poliovirus is not inactivated by neutral red and light in the absence of cells, but virus grown in cells containing neutral red may be inactivated by light, indicating that an incorporation of the dye into developing poliovirus may occur. We have found that acridine orange yields similar results, and as described below, have obtained direct microscopic evidence of the incorporation of the dye into the virus.

Experiments performed with proflavin and other acridines as viral inhibitors have been carried out, until recently, without reference to the part that light may play in this inhibition. The possibility of incorporation of the dyes into the virus particle and its subsequent inactivation by light was not considered by previous workers. Thus Ledinko[7] demonstrated the cytopathogenicity of poliovirus to be unaffected by proflavin, and we have observed that the same is true using neutral red, unless near-toxic levels of dye are used. Both proflavin and neutral red are capable of inhibiting the production of infective poliovirus even in subdued lighting. It is unknown as yet exactly what part the dye alone and what part added light play in the production of non-infective virus. In the experiments of Ledinko and Schaffer, virus produced in the presence of proflavin, and shown by Schaffer to contain the dye, was largely non-infective, but the effect of laboratory lighting was not excluded. In later experiments carried out in the dark, Schaffer has also found that proflavin produces only slight inhibition in the production of infective poliovirus (personal communication).

CYTOCHEMICAL AND FLUORESCENT ANTIBODY STUDIES ON THE GROWTH OF VIRUSES

Virulent and attenuated polioviruses. In the past few years two versatile techniques have added much to our knowledge of the intracellular localization of viruses and of their cycles of development: one is the fluorescent antibody method of Coons and Kaplan[9] which locates specific viral antigens, the other is the acridine orange technique developed independently by Armstrong[9] and by von Bertalanffy and Bickis,[10] a technique which can be used as a cytochemical method for demonstrating intracellular nucleic acids. A number of investigators have used one or the other of these techniques in studies of host-virus relationships. For fuller citations of the literature, see other references.[11, 12]

Recently, interest has been focused on the effect of vital, photodynamically active dyes (of which acridine orange is an example) on the infectivity

of virus particles. The action on both mature and developing particles has been studied. LoGrippo and Basinski[13] have shown that the whole poliomyelitis particle, in contrast to the T3 bacteriophage, is not inactivated by the action of acridine orange and ultraviolet light. Hiatt[14] has demonstrated that the enteroviruses are unaffected by exposure to toluidine blue and light, while herpes B, vaccinia, rabies, simian vacuolating viruses, and, to a lesser extent, adenoviruses and reovirus, *are* inactivated. As noted above and in a previous report,[1] mature poliovirus incubated with neutral red and exposed to white light showed no reduction in titer, but virus *grown* in cells containing the dye was inactivated by exposure to light, suggesting incorporation of the dye into the virus particle. Schaffer[6] obtained direct evidence for the incorporation of proflavin into poliovirus during its growth in tissue culture and assumed that the dye becomes bound to the RNA of the virus particle. Our experiments described below are an attempt to present an integrated cytochemical and biological study on the developing poliovirus particle in tissue culture.

MATERIALS AND METHODS

The sequence of changes in coverslip tissue culture monolayers of monkey kidney cells infected with poliovirus (virulent and attenuated strains) has been followed by cytochemical means (acridine orange staining) and by the fluorescent antibody technique. Changes have been followed closely during a single cycle of infection and beyond, both at 37°C and at 30°C. In Carnoy-fixed preparations, stained with 0.01% acridine orange at pH 4.0, material identified as DNA stained a brilliant greenish-yellow and was susceptible to DNAase; material identified as RNA stained a brilliant flame red and was susceptible to RNAase. Techniques in current use in our laboratory for controlled nuclease digestion tests have been described together with our method for growing poliovirus in the presence of acridine orange.[15] The direct fluorescent antibody procedure[10] using immune monkey globulin conjugated with fluorescein isothiocyanate was employed. Reichert fluorescence equipment and suitable filters were used.

RESULTS

In general, no significant differences between the virulent and attenuated strains of poliovirus could be detected with acridine orange or by the fluorescent antibody technique. When incubated at 37°C. virus protein (antigen) and increased cytoplasmic RNA were detectable three hours after infection and both reached a maximum in staining intensity about six to eight hours after inoculation. At no time was specific antigen detected in the nucleus of infected cells, although marked nuclear alterations and enhanced nucleolar staining were noted by the acridine orange technique three hours after inoculation. At eight hours, evidence of virus

release was observed with both techniques. Figures 2, 3, 5 and 7 show some of our observations with these techniques. Parallel titrations of intracellular virus (Fig. 8) revealed a gradual increase in the amount of virus which reached a maximum at six to eight hours, in excellent agreement with the cytochemical and fluorescent antibody findings.

Similar results were obtained at 30°C., but intracellular changes occurred approximately four hours later than those observed at 37°C. The peak of staining intensity occurred approximately twelve hours after inoculation. In contrast to growth at 37°C., very little virus release was observed at 30°C. (Fig. 6) at any time. However, release could be obtained from 30°C. cultures by placing them at 37°C. for an additional hour at the end of the incubation period. Actually, a brilliant picture of virus release was then observed (Fig. 4). Figure 8 shows the slower growth rate at 30°C., in agreement with the cytochemical and fluorescent antibody findings. This figure also shows titers of intracellular virus which were reached in the presence of acridine orange. The small amount of inhibition found was probably due to photodynamic inactivation by stray light.

DISCUSSION

Fluorescent antibody and acridine orange techniques are both highly sensitive means for following the growth of poliovirus in tissue culture. The limit of resolution of the fluorescent antibody technique is in the vicinity of 5×10^{-15} gm. antigen,[16] which corresponds to some hundreds of poliovirus particles. Mayor and Diwan[15] have estimated the sensitivity of the acridine orange technique for small RNA viruses to be of the order of 50 particles. However, the specificity of the acridine orange method —particularly for the study of RNA viruses against a background of cellular RNA—is dependent on changes in density and distribution of the nucleic acid and is not as highly specific as the fluorescent antibody technique.

Anderson *et al.*[17] have pointed out that in the case of DNA viruses a relative insusceptibility to DNAase digestion is a constant feature of animal virus infections and that resistance to the nuclease could be abolished by pretreatment with a proteolytic enzyme. Here we have a useful tool for distinguishing between the host cell DNA and the viral DNA. However, so far no cytochemical method has been found for distinguishing between viral and cellular RNA. Influenza,[17] Rous sarcoma,[18] and poliovirus,[19] are RNA viruses which in the fixed state have proved susceptible to RNAase. Perhaps a method of fixation which will allow digestion of cellular RNA while leaving viral RNA intact will be developed.

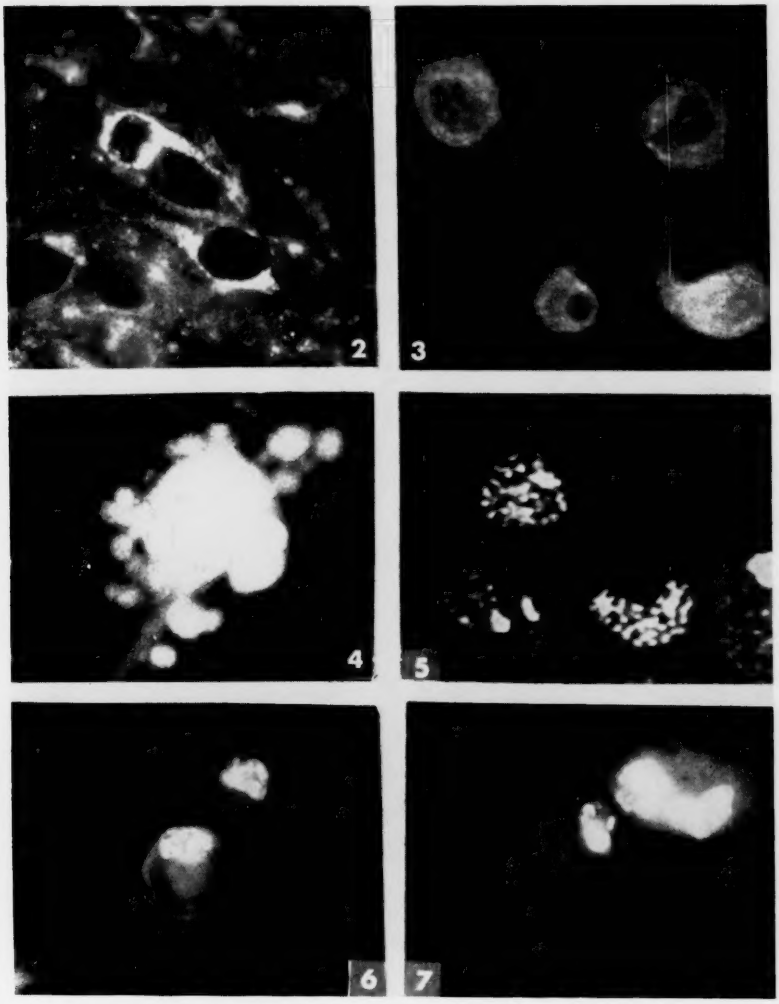

FIG. 2. Monkey kidney cells 3 hours after infection with LSc type 1 attenuated poliovirus stained by the direct fluorescent antibody technique for viral antigen. Note bright fluorescent staining in a ring around the nucleus in 2 cells at the center of the field. X1,000

FIG. 3. Monkey kidney cells 8 hours after infection with LSc type 1 attenuated poliovirus, incubated at 30° C. Note swelling of cells, integrity of nucleus and diffuse antigen. X1,000

FIG. 4. Monkey kidney cells 21 hours after infection with MEF_1 type 2 virulent poliovirus, incubated 20 hours at 30° C. and then placed for 1 hour at 37° C. Note intense fluorescence completely filling the cell and extensive release of antigen. X1,200

FIG. 5. Monkey kidney cells 3 hours after inoculation with Y-SK type 2 attenuated virus. Nuclear clearing and clumping of DNA are evident. Nucleoli are stained bright red. X2,000

FIG. 6. Monkey kidney cells incubated at 30° C. for 8 hours after inoculation with Mahoney type 1 virulent poliovirus. Note three dimensional appearance of cells and peripheral RNA staining; also absence of virus release. X900

FIG. 7. Monkey kidney cells 8 hours after inoculation with LSc type 1 attenuated virus. Note rounded cells full of RNA and eccentric brilliantly stained nuclei. Release of RNA material is evident at the cell walls. X2,000

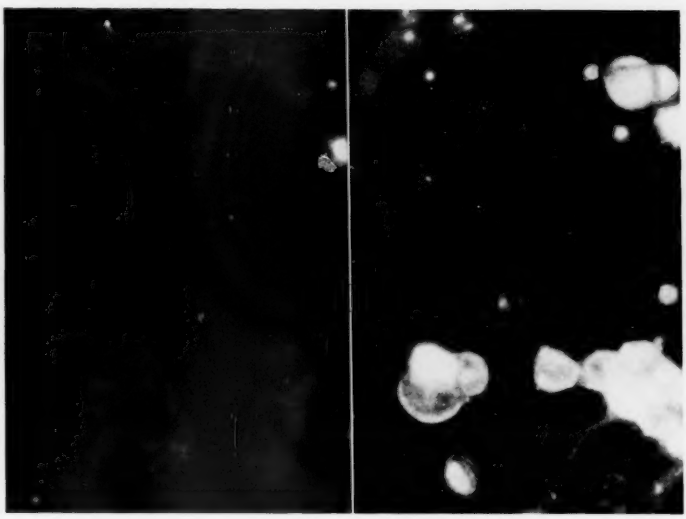

FIG. 9. Eight-hour yields of type 2 poliovirus stained with fluorescent antibody. Photomicrograph left: virus culture in presence of guanidine. Photomicrograph right: virus-infected cells in control culture.

Inhibitory action of guanidine on poliovirus multiplication. Guanidine, $(NH_2)_2CHN$, is an effective inhibitor of poliovirus multiplication in monkey kidney cell cultures.[20] As little as 60 μg per ml. of guanidine-HCl will reduce the yield of virus by five logs. Extensive studies on the mode of action of guanidine have been carried out in our laboratory in an attempt to elucidate the site at which the compound acts in the virus multiplication cycle.[21] The inhibitor prevents cytopathic changes in infected cultures and interferes with the production of infective polio-

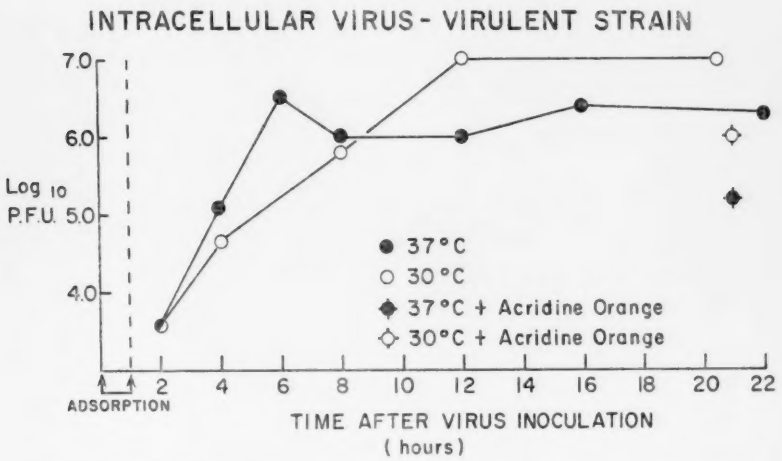

FIG. 8. Growth of poliovirus type 2, MEF_1 virulent strain, at 37° C. and at 30° C. Titers reached in presence of acridine orange after 21 hours at 37° C. and 30° C. are included.

virus. Staining with acridine orange and with fluorescent antibody proved useful in determining that guanidine can inhibit both the production of cytoplasmic RNA associated with poliovirus infection, and the production of antigenic viral protein.

The following experiment was carried out to elucidate further the nature of guanidine inhibition of poliovirus multiplication. Monkey kidney cells were grown on coverslips in Leighton tubes. Three sets of such cultures were used in each test: into one set, virus alone was inoculated; into another, virus plus 100 μg of guanidine; a third set, without virus and without guanidine, served as controls. The virus inocula consisted of 0.1 ml. of undiluted poliovirus Type 2 MEF_1 (titer $10^{8.1}$ PFU/ml.). One hour was allowed for adsorption after which excess virus was washed off.

After five hours, tubes were harvested for titration and several were stained with acridine orange. After eight hours, additional tubes were harvested and several were stained with fluorescent antibody, using the direct method.

The results obtained are shown in Table 2. When stained for antigen by the fluorescent antibody technique, controls were uniformly negative. Cultures of virus grown in the presence of guanidine in monkey kidney cells were also negative. However, virus grown for eight hours showed bright fluorescence (Fig. 9), but prior treatment of the cells with specific antiserum inhibited the staining. These experiments indicate that the antigenic protein coat of the virus was not being formed under guanidine inhibition of infective virus.

TABLE 2. GUANIDINE INHIBITION OF TYPE 2 POLIOVIRUS

	Control		*100 μgm./ml. guanidine*	
Time of harvesting	5 hrs.	8 hrs.	5 hrs.	8 hrs.
Log PFU titer	6.7	7.2	3.8	4.1
Log inhibition	—	—	2.9	3.1

In cell controls similar to those in the fluorescent antibody staining experiment, acridine orange staining showed a uniform but not intense red staining of the cytoplasm. Cells containing virus grown under normal conditions showed an intense red staining of the cytoplasm suggesting an active production of RNA over that of controls, as described above. Cells containing the small amount of virus which developed in the presence of 100 μg of guanidine for five hours showed the same pale red staining as the controls, suggesting that no marked change in cytoplasmic RNA had taken place under the conditions. It follows from these experiments that guanidine inhibits both the production of the protein antigen of the virus and the increase of red-staining cytoplasmic RNA associated with viral synthesis.

Guanidine is a remarkable inhibitor of poliovirus multiplication in that structurally it is one of the simplest molecules capable of inhibiting the development of a virus. Substitution of any of the nitrogen groups yields an inactive molecule, indicating the specific nature of its action.[31]

ACRIDINE ORANGE STAINING OF PURIFIED VIRUSES

Poliovirus and tobacco mosaic virus (RNA). The ability to see, measure, and count the actual elementary bodies of viruses is funda-

mental from a biophysical standpoint and necessary for their identification or for quantitative correlation with their biological effects. Although some of the large viruses, such as vaccinia, can be identified under the light microscope with suitable staining, and the filamentous forms of influenza virus are readily visible by dark field illumination, it is generally necessary to employ electron microscopy to determine shape and to measure directly the size of most virus particles.

Using the acridine orange staining technique, Anderson, *et al.*[17] were able to identify individual heads of T_2 bacteriophage particles with the fluorescence microscope. As the DNA content of T_2 bacteriophage is about 2×10^{-10} μgm per particle,[22] this finding represents a high degree of sensitivity for the detection of DNA by cytochemical methods. Anderson, *et al.* were unable to detect individual RNA virus particles and considered their identification beyond the limit of sensitivity of the technique. Epstein and Holt[18] examined purified preparations of Rous sarcoma virus in the fluorescence microscope and established RNA as the sole nucleic acid present. Correlated studies in the electron microscope localized the RNA in the nucleoid of the virus particle. It is obvious that the use of the fluorochrome, acridine orange, as a vital dye presents an intriguing possibility for visualizing individual virus particles by virtue of their vitally stained nucleic acids. We have carried out experiments on these lines with purified poliovirus and tobacco mosaic virus.

MATERIALS AND METHODS

Purification and concentration of virus. A single procedure was followed for both poliovirus and AO-poliovirus (grown in the presence of acridine orange) except that in the latter case maximum precautions were observed at every stage of the process to protect the infective fluids from inactivation by light. The virus pools were clarified by low speed centrifugation and concentrated by overnight treatment at 4°C with polyethylene glycol (Carbowax 20 M). Aliquots of the virus suspensions, each volume approximately 70 cc., were then purified and further concentrated chromatographically by adsorption to and elution from a calcium phosphate column. Final concentration was achieved by ultracentrifugation.

Studies of tobacco mosaic virus (TMV) were carried out on a sample of purified concentrate prepared and sent to us in 1943 by Dr. Wendell M. Stanley.

Staining technique. Small droplets of virus concentrates were placed at the center of 11 x 22 mm. coverslips and allowed to dry thoroughly in air. When desired, fixation was carried out on these previously dried preparations. Fixation with chilled absolute methanol for 15 minutes was found most satisfactory. In an additional experiment the following concentrations of methanol were used: 95%, 70%, 50%, 40%, 30%. Carnoy's fluid was also employed as a fixative in a number of cases. The standard procedures for acridine orange staining and for nuclease digestion used in this laboratory[11,15] were employed. The staining was with 0.01% dye at pH 3.8-4.

RESULTS

The methods followed for purification and concentration of virus were found to be entirely satisfactory. Electron microscopy showed that the poliovirus concentrates consisted entirely of spherical particles approximately 30 mμ in diameter while the TMV concentrates yielded typical rods. Table 3 gives the titers of virus (in PFU per ml.) obtained for poliovirus and AO-poliovirus before and after the purification and concentration process. In each experiment the final volume, after ultracentrifugation, was approximately 1/100 the volume of the original tissue culture fluid. Table 3 shows clearly that in the case of poliovirus the log increase in titer of

TABLE 3. TYPICAL TITERS OF POLIOVIRUS AND AO-POLIOVIRUS FRACTIONS

Material	*Poliovirus* $\log_{10}$ *PFU/ml.*	*AO-Poliovirus* $\log_{10}$ *PFU/ml.*
Original tissue culture fluid	8.5	7.7
After purification by Carbowax, chromatography, and ultracentrifugation	10.3	9.2

1.8 over the whole procedure is in excellent agreement with the concentration data, and that very little virus has been lost. In the case of AO-poliovirus with a log increase in titer of 1.5 after concentration, there may have been a small amount of photodynamic inactivation during the course of the purification and concentration procedure.

Staining properties of purified RNA virus concentrates. The final poliovirus concentrates, when fixed and stained with acridine orange, presented the brilliant flame-red fluorescence characteristic of RNA. These fixed preparations were completely susceptible to RNAase indicating that only RNA material was present. No other staining colors were visible in any of these preparations. The TMV concentrates also showed brilliant RNA staining, in the fixed state, again susceptible to RNAase.

A summary of the purified RNA virus staining properties with acridine orange is shown in Table 4. Of particular interest are the results obtained with the fluorescence microscope using unstained concentrates. While poliovirus and TMV exhibited no staining at all under these conditions, the AO-poliovirus concentrates fluoresced a brilliant apple-green, indicating directly that acridine orange fluorochrome had been incorporated into the virus particle. After alcohol fixation, staining was even more pronounced, indicating that dye binding was not just a surface phenomenon in which

the strongly basic acridine orange molecules might be bound to the surface of the negatively charged virus particles. Such an effect would be expected to be nullified by alcohol fixation.

RNA viruses, such as TMV or poliovirus, fluoresce with a brilliant red, but poliovirus grown in the presence of the fluorochrome displays a unique, brilliant, apple-green fluorescence. It is known that basic dyes, such as acridine orange, are bound to nucleic acids by ionic bonds to the phosphate groups, and possibly by weaker Van de Wall's forces. If dye molecules

TABLE 4. STAINING PROPERTIES OF RNA VIRUS CONCENTRATES

	Poliovirus		*TMV*		*AO-Poliovirus**	
Stain	*Unfixed*	*Fixed*	*Unfixed*	*Fixed*	*Unfixed*	*Fixed*
None	No Fluorescence	No Fluorescence	No Fluorescence	No Fluorescence	Brilliant Apple Green	Brilliant Apple Green
.01% A.O.	Pale Green	Brilliant Flame Red	Brilliant Apple Green	Brilliant Flame Red	Brilliant Apple Green	Brilliant Flame Red
.001% A.O.	Pale Green	Brilliant Apple Green	Brilliant Apple Green	Brilliant Apple Green	Brilliant Apple Green	Brilliant Apple Green

* Grown in the presence of 0.0005% acridine orange.

were in proximity to the sugar surface of a nucleic acid molecule, this is sterically possible. So too, in considering the incorporation of acridine orange into maturing poliovirus particles concentrations of the dye in the medium must be taken into account. Schümmelfeder[28] has shown that for fixed tissue at high concentrations of dye (0.05%), cells demonstrated in all parts a change from green→ yellow→ orange→ red with rising pH. With sufficiently low concentrations (0.01%) the cytoplasm (RNA) alone showed a change to red, the nucleus (DNA) remaining yellow even at high pH. The bright green fluorescence of our incorporated concentrates with an RNA virus indicates a low concentration of the dye. We can also conclude that the steric configuration of the RNA molecule makes it possible for larger quantities of the fluorochrome to become attached during the staining process, leading to the development of red fluorescence while under standard conditions the DNA molecule, only, is capable of binding sufficient dye to yield yellow fluorescence.

When the virus concentrates were stained with 0.01% acridine orange at pH 3.8 according to the standard procedure, the following results were obtained. Fixed preparations of poliovirus yielded the brilliant red fluorescence characteristic of RNA; on the other hand, unfixed ones gave the very faintest green indicating either surface binding of the strongly positively charged fluorochrome to the protein of the unfixed virus surface, or limited penetration of the virus particle by the dye. Fixed preparations of TMV also showed red RNA staining, while unfixed preparations, when allowed to remain in contact with the dye solution for some hours, showed a green fluorescence identical to that of the unstained AO-poliovirus. It would appear that, in contrast to poliovirus, the unfixed TMV particle is permeable to acridine orange and that a sufficient concentration of the dye can accumulate within the particle to develop a green fluorescence. The brilliant green color exhibited by the "vitally stained" AO-poliovirus in the unfixed state was not altered by subsequent staining with 0.01% acridine orange. This would be the expected result if the mature, unfixed poliovirus particle could not be penetrated by the fluorochrome. However, the fixed, and subsequently stained, AO-poliovirus concentrates did exhibit a flame-red, RNA fluorescence, as would be expected. These results are listed in Table 4.

The results obtained when staining was carried out with 0.001% acridine orange are also shown in Table 4. With this lower concentration of stain it was impossible to develop the characteristic red RNA color seen with standard staining conditions (0.01%AO) even in fixed preparations. This concentration effect is of particular interest when one considers that optimal conditions for the growth of AO-poliovirus *in vitro* require a concentration of fluorochrome as low as 0.0005%. At this low dye concentration it is impossible to grow mature, infective AO-poliovirus particles exhibiting a typical red RNA fluorescence.

Behavior of RNA virus concentrates after treatment with ribonuclease. The specificity of the RNA staining of all the concentrates studied was established by digestion with ribonuclease. Fixed preparations of poliovirus, AO-poliovirus and TMV were readily susceptible to the enzyme while control virus specimens retained their ability to develop a red fluorescence on subsequent staining. Dried, unfixed preparations of TMV were completely insusceptible to RNAase activity while similar poliovirus preparations were usually susceptible. This is to be expected when one considers the extreme lability of the dried poliovirus particle.

The behavior of fixed preparations of AO-poliovirus with ribonuclease provided additional strong direct evidence that the incorporated vital dye

is, in fact, associated with the nucleic acid moiety of the developing virus particle. The apple green fluorescence of these preparations was almost completely extinguished after incubation with RNAase. This effect was not destroyed by proteolytic enzymes and incubation of similar preparations in the vehicle alone had no effect on these concentrates.

Permeability studies on poliovirus and tobacco mosiac virus. It was possible to carry out a "titration" of the virus surface permeability by staining with acridine orange after fixation in various concentrations of methanol. In the case of poliovirus, fixation and penetration were complete, and red staining developed with concentrations of methanol greater than 70%. Below this methanol concentration, denaturation of the protein coat was not sufficiently extensive to permit penetration of the fluorochrome, and no staining developed. The methanol end point for TMV was slightly lower than that for poliovirus but the general effect was the same. Our experiments with acridine orange demonstrate directly that this vital dye is incorporated into the virus particle. Removal of the characteristic staining with ribonuclease indicates that the dye is, indeed, bound to the nucleic acid of the virus.

As concentrations of acridine orange in excess of 0.003% were highly toxic to tissue culture cells even in the dark, our experiments were carried out in very dilute dye solutions (0.0005%). Even if all of the available dye were to react with the polynucleotide chains of developing virus particles, only minute quantities of dye, incapable of giving a typical red RNA color, would be present despite large aggregates of close-packed particles. Also, from purely physical considerations, it is unlikely that the developing poliovirus particle could incorporate sufficient dye to exhibit other than a green fluorescence. The large quantities of dye required to yield red fluorescence would probably preclude the formation of mature, infective virus particles because of steric hindrance. It would not be possible to form a protein coat around acridine orange-RNA aggregates of such great size. A comparison can be made with TMV, which is definitely permeable to acridine orange. The unfixed TMV particle, with protein coat intact, is penetrable only by sufficient dye to develop a green fluorescence. Hiatt[14] has inferred by direct means that many viruses (e.g., T_3 phage, vaccinia, rabies) are permeable to vital dyes while others (enteroviruses, reoviruses) are completely impermeable. His interpretations are in complete agreement with the results of our direct staining.

Bacteriophage ΦX174, (*Single-Stranded DNA*). Recently there has been a growing interest in a group of small bacterial viruses of which ΦX174 is a member. Hall, *et al.*[24] examined ΦX174 in the electron

microscope and found tail-less particles of 248 Å diameter. They had polygonal outlines consistent with the icosahedral symmetry postulated for many "spherical" plant and animal viruses. Tessman[25] studied inactiviation rates of the same virus by the decay of incorporated radioactive P-32 atoms and obtained an efficiency of inactivation ten-fold greater than those observed with other bacteriophages. He was of the opinion that this indicated the presence of an unusual DNA, possibly a single-stranded form. Sinsheimer[26,27] carried out extensive physical and chemical studies on highly purified preparations of the virus particle and its DNA. The DNA reacted rapidly with formaldehyde, exhibited high flexibility in light scattering experiments, and lacked a complementary nucleotide structure. These findings and the kinetics of enzymatic degradation (by pancreatic deoxyribonuclease) were all consistent with the existence of a single-chain polymer.

We had concluded, from the experiments discussed above, that dye-binding was a matter of steric-fit, and that the color of the emissions from stained viruses depended on dye concentration, not on the chemical differences between nucleic acids. Because the color-shift is not a function of chemical composition, it seemed worthwhile to study the staining reaction of the anomalous nucleic acid in ΦX174.[28] Chemically, it is DNA but its single-strand configuration endows it with unusual physical properties. In our experience two bacteriophages were studied, ΦX174 and T_2. Droplet preparations were fixed, stained with 0.01% acridine at pH 3.8, and examined in blue-violet light. Both DNAase and RNAase were used to check the specificity of staining reactions, and control specimens were incubated in the vehicles for the enzymes.

RESULTS

ΦX174 fluoresced a brilliant flame-red in dried droplet preparations. The fluorescence was destroyed completely when fixed preparations were subjected to DNAase digestion before staining but not when RNAase was used. Incubation of specimens in the enzyme vehicle alone, had no effect on the development of the red fluorescence. Samples of purified T_2, processed at the same time, yielded the typical yellow-green fluorescence of standard DNA.

DISCUSSION

The acridine orange staining properties of bacteriophage ΦX174 differ from those of any other virus previously studied by this method. Red fluorescence at pH 3.8 is usually associated with RNA. In the case of

ΦX174, however, the red fluorescing substrate was destroyed by DNAase but not by RNAase. The apparent paradox of a red-fluorescing DNA is suggestive of a DNA molecular configuration similar to that of RNA, namely, a single-stranded DNA.

It is not necessary to subject preparations of ΦX174 to pre-treatment with a proteolytic enzyme for DNAase to be effective. This is in agreement with the behavior of large bacterial viruses and in contrast to that of the DNA animal viruses in which a close association between the nucleic acid of the virion core and the protein capsid appears to exist.

TABLE 5. STAINING PROPERTIES OF VIRUSES

Virus preparation nucleic acid	*Color developed on acridine orange staining*	*Nuclease susceptibility*		
		DNAase	*RNAase*	*Proteolytic enzyme necessary*
Double-stranded bacteriophage DNA	yellow green	+	—	—
Double-stranded animal virus DNA	yellow green	+	—	+
Animal virus RNA	flame red	—	+	—
Single-stranded bacteriophage DNA	flame red	+	—	—

Schümmelfeder *et al.*[30] reported that concentration of the dye played an important part in the development of fluorescence and that the highly polymerized condition of the DNA molecule prevented intracellular DNA structures from developing a red color, even under conditions of favorable pH. They noted, also, that DNA structures tended to form reddish-fluorescing complexes after acid hydrolysis. We have stressed the importance of the steric arrangement of the DNA and RNA molecules and suggested that RNA should have many more exposed sites for the attachment of acridine orange molecules, a condition favoring the developing of red fluorescence.[15] Similar steric possibilities must exist in the case of the DNA of bacteriophage ΦX174. It would appear that the acridine orange technique, controlled by suitable nuclease digestion tests, is a simple and direct method of detecting DNA in the single-stranded form. It may have applications at the cellular level of virus reproduction.

The existence of a single-stranded DNA molecule as an entity, as in bacteriophage ΦX174, focuses attention on the general mechanisms of DNA replication. Recent results with bacteria and plants are in agreement with the concept of DNA replication by separation of the two chains of the double helix. Whether this results in a complete separation of the strands to a single-stranded state and formation of separate templates is still a matter for speculation; however, it is known that the single-stranded ΦX174 is an excellent primer for the synthesis of a double-stranded DNA with the enzyme *E. coli* polymerase.[30] The fact that

TABLE 6. CsCl Isodensity Purification of Polyoma and Adenovirus

	*Polyoma virus**			*Adenovirus***	
Virus material	*Volume ml.*	*HA/ml.*	*PFU/ml.*	*Volume ml.*	TCD_{50}/*ml.*
Original fluid	30	160	10^7	80	$10^{8.5}$
Pellet	1.5	640	$10^{7.4}$	1.5	$10^{5.5}$
CsCl band	1	80	$10^{6.4}$	1	$10^{5.0}$

* Assays kindly carried out by Dr. Matilda Benyesh-Melnick.
** Assays kindly carried out by Mr. E. Seidel.

ΦX174 multiplies readily in *E. coli* yielding infective single-stranded virus particles would presuppose a single→ single-stranded mechanism for DNA replication. From the above results and those of a number of other laboratories, it is possible to set up a simple table to serve as a means for identifying the type of nucleic acid present in different viruses using their staining reactions with acridine orange (0.01% pH 4) and their behavior with specific enzymes (Table 5).

Polyoma Virus and Adenovirus (DNA). Recently attention has been focused on the nucleic acids of polyoma virus[31, 32] and adenovirus.[33, 34] The bulk of experimental evidence indicates that both are DNA viruses. In addition, cytochemical studies with polyoma[35, 36, 37] and adenovirus[38, 39, 40] show that a marked increase occurs in nuclear DNA during the replication of these viruses but it is not known how much of the DNA is actually virus, or if it exists in the single or double-stranded form.

We have purified both viruses by CsCl isodensity centrifugation and have studied the staining reactions of preparations with known virus activity (Table 6). Droplet preparations for acridine orange staining and nuclease digestion tests were made from the CsCl bands dialysed free from the salt. It was a simple matter to place both polyoma and adenovirus

in their correct position in Table 5. The behavior of both viruses was consistent with that of double-stranded, animal virus DNA. It may be argued that any DNA virus replicating in the nucleus could adsorb sufficient cellular DNA to its surface to give a double-stranded color reaction in the standard acridine orange test. However, an additional pre-fixation treatment with DNAase which would certainly remove any contaminating cellular DNA from the preparations, did not interfere with the subsequent development of the specific fluorescence.

This technique of staining CsCl—banded viruses could become a relatively simple test for determining the type of nucleic acid present in known viruses as well as in new agents. Determining information of the nucleic acid in a given virus is required for purposes of classification and for improving our understanding of virus replication.

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J. N. MORRIS*

Social Medicine Research Unit,
Medical Research Council
The London Hospital, London, England.

EPIDEMIOLOGICAL ASPECTS OF ISCHAEMIC HEART DISEASE†

FOREWORD

THIS paper was read at the Symposium on World Medicine during the Sesquicentennial of the Yale University Medical School, 17 March 1961. I am proud to have it included in this tribute to John R. Paul whose friendship I am privileged to enjoy and from whose learning and wisdom I have so much benefited.

ISCHAEMIC HEART DISEASE — A WORLD PROBLEM

Ischaemic heart disease is very unevenly distributed throughout the world, and one of the few facts concerning its etiology is that populations with low standards of living suffer from it far less than do populations with high standards of living. Nevertheless, the disease is of concern to all countries, since it seems to follow inexorably in the wake of industrial and economic advances. In fact, India has been one of the leading proponents of its study by the World Health Organization.

In the United States of America ischaemic heart disease (IHD) is often called Public Health Problem No. 1. Its incidence among middle-aged men in Framingham, Massachusetts, is a little more than one percent per year.[1] Thus, if this figure is typical, very little arithmetic is required to show that one of every three or four Americans suffers a clinical episode of IHD in middle age. Judging from mortality data,[2] the experience in Sweden is much better, the rate there being only about one-third that in the United States, while the rates for England fall somewhere between these two. The wide range of IHD found in the highly developed countries offers hope for its eventual control short of abolishing western civilization.

In any consideration of IHD, it is necessary to remember two sets of facts which have now been established. The second set cannot be related with any confidence to the first, nor do the two together provide more than a woefully inadequate picture of pathogenesis. Nevertheless, an important lesson emerges.

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*Director and Professor of Social Medicine in the University of London. Formerly visiting Professor of Epidemiology in Section of Epidemiology and Preventive Medicine and Department of Public Health, Yale University School of Medicine 1957-1958.

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(i) *Coronary atheroma and coronary occlusion.* The basic pathology of the disease consists of the presence of atheroma or atherosclerosis in the walls of the coronary arteries, together with narrowing or closure of the vessel lumina by an intravascular thrombosis and its organization. The more the walls of the arteries are affected by atherosclerosis, the more likely is occlusion to occur. However, epidemiological evidence agrees with clinical experience and laboratory experiment that occlusion is by no means a simple function of the amount of atherosclerosis or the extent of atheroma.[3, 4] It follows that coronary occlusion and coronary atheroma have different causes; and since for the development of IHD, both are necessary, IHD must have multiple causes. There can not be a single or simple *cause* of IHD like saturated fat, or lack of exercise, or "nervous strain".

(ii) *High blood cholesterol, high blood pressure, and IHD.* The principle findings of studies conducted in Framingham, Massachusetts,[1] in Albany, New York,[5] and elsewhere, are that hypercholesterolemia and hypertension are harbingers of IHD. The higher the levels of these two, the greater the risk of IHD. Very little IHD occurs in the absence of either, but when both are present, the incidence may rise to 4% *per year.* Both in the Framingham study, and in our investigation of London busmen,[4] hypertension and high serum cholesterol levels were found to be fairly independent of one another. That is to say, individuals with elevated blood pressure did not show a particular tendency to elevated serum cholesterol levels, nor vice versa. Here again, the existence of two independent factors, each of which bears some relation to the pathogenesis of IHD, implies a multiplicity of causes of the disease.

Clearly, then, many things are involved in the production of IHD, a conclusion supported by clinical experience. Death coming "out of the blue" to the younger man, or to the post-operative middle-aged individual, or to the old man shovelling snow or during a smog; the infarction in the ovariectomised young woman, in the middle-aged diabetic, or in the old lady with ruptured heart; these and many more syndromes have accustomed the clinician to think in terms of a "multifactorial" situation. Even if it turns out that one cause is *necessary* (perhaps one relating diet to thrombosis) there is no indication that this will be any more *sufficient* an explanation of the etiology of the disease than is Koch's bacillus in tuberculosis, or the hemolytic streptococcus in rheumatic fever.[6a]

POSSIBLE CAUSES IN THE WAY OF LIFE

Recently attention has been focussed on habits and patterns of living which might be related to the increasing incidence of ischaemic heart disease.

In particular, the possible roles of exercise, of nervous tension, and of diet have been investigated.

Exercise. There is now considerable evidence that physically active workers have less, and less severe, IHD than do light workers. This fact is interesting in view of two trends: first, the rising incidence of IHD; and second, the decrease in amount of exercise. Reduction of physical activity is surely one of the characteristic social changes of the present century, and automation promises to finish the job. The correlation between sedentary occupation and high attack rates of IHD have now been made

TABLE 1. HEALTH OF WORKING LONDON BUSMEN[4]

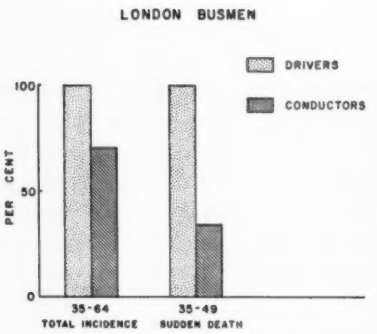
Ages of men	*Percentage plasma cholesterol* 270 mgm. and over*		*Percentage casual diastolic B.P. 106 and over*		*Percentage three standard skinfolds 60 mm. and over*	
	Drivers	*Conductors*	*Drivers*	*Conductors*	*Drivers*	*Conductors*
40-49**	(22)†	(18)	4.2	0	(15)	(9.7)
50-59	32	17	14	6.2	22	10
60-64	37	14	(14)	(11)	24	5.6

* Tests performed by Courtauld Institute of Biochemistry, Middlesex Hospital.
** Age-Occupation Groups of 39-187.
† Parentheses indicate that differences are not significant at 5% level.

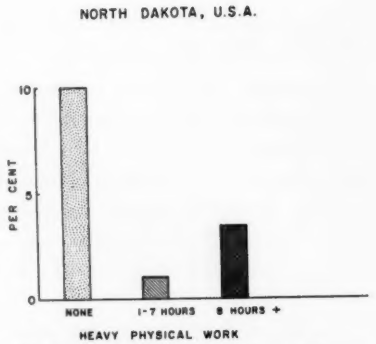
in several countries, including the United Kingdom,[3, 4] United States,[7] Israel,[8] Poland,[9] Finland,[10] Russia,[11] (Fig. 1). It should be noted, however, that active workers still have too much IHD and clearly if lack of physical activity is a cause, it is of different importance in different settings. For example, in California[12] (Fig. 1) there appears to be no occupational activity effect at all; or is it, perhaps, overwhelmed by other factors?

These observations on physical activity can be taken further. In terms of the main precursors of IHD discussed above, such as serum lipids, blood pressure, and of another possibly related factor, obesity, the light workers are clearly at a disadvantage, as shown in Table 1. The differences between sedentary drivers and physically active conductors, for example, are more than sufficient to account statistically for the differences in the incidence of IHD in these two groups.

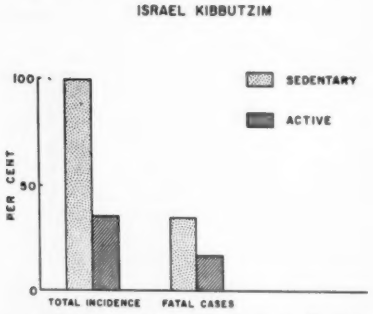
Several related questions arise. Manifestly, different men select different jobs. To what extent are the differences shown in Figure 1 and Table 1 the result of differences which the men brought with them to their jobs? And to what extent do the changes in physiology and pathology arise



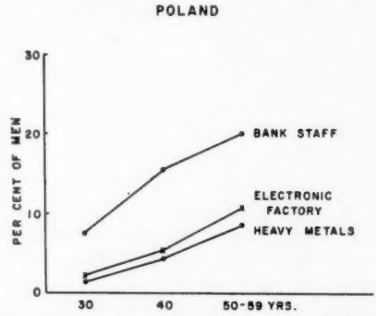
a) Incidence of coronary heart disease, 1949-1958.



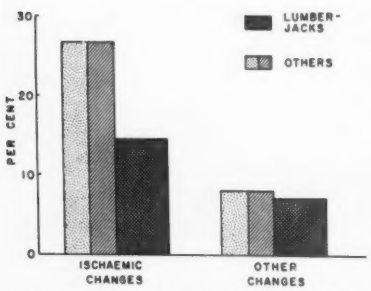
b) Incidence of coronary heart disease, 1957. Men aged 35-54. From Zukel, *et al.*[7]



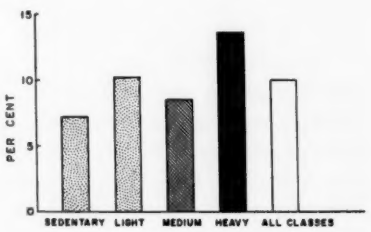
c) Incidence of coronary heart disease. Average annual rates per 1,000 men 40-65, 1949-1958. From Brunner & Manelis.[8]



d) Prevalence of coronary heart disease. From Aleksandrow.[9]



e) Prevalence of ECG abnormalities, males aged 40-59. From Karvonen, *et al.*[10]



f) Incidence of coronary heart disease, civil servants aged 40-70. From Chapman, *et al.*[12]

FIG. 1. Ischaemic (Coronary) Heart Disease and Physical Activity of Occupation.

because, having selected a particular occupation, the men then spend 10, 20, or 30 years doing different things? This question is, of course, intimately connected with the next:—What are the implications for preventive medicine? Clearly, if the constitutional factors of inheritance and the influence of early environment are mainly responsible for the increase in IHD, there will not be much benefit from prophylactic programs of mass physical culture such as the Russians and Chinese have now launched.

These questions illustrate the problem of elucidating the nature of the various factors involved in the development of chronic disease during middle and old age; inherited, historical, and current internal and en-

TABLE 2. BLOOD LIPIDS, OCCUPATION AND FAMILY HISTORY[4]
Men Ages 40-64, London Transport Executive

	Drivers		*Conductors*	
Mgm. %†	*Parent dying middle age* (122)	*Parents living to old age* (141)	*Parent dying middle age* (90)	*Parents living to old age* (62)
Beta lipoprotein	206*	193*	190**	184**
Sf 100-400‡	69*	45*	36**	27**

* Significant test, $.02 > p > .01$.
** Not significant.
† Tests performed by Courtauld Institute of Biochemistry, Middlesex Hospital.
‡ Low density lipoprotein fraction.

vironmental factors must be evaluated. In relation to IHD, there are a few hints of what might be happening. It is evident that London bus drivers and bus conductors have different physiques from the beginning of their careers in London Transport.[4] However, this does not explain away the differences between them in the incidence of sudden death; thin drivers have higher rates than thin conductors, etc. In regard to serum lipids, two kinds of factors can be seen to operate (Table 2) if it is granted that premature death of a parent implies a bad family history. Among the drivers, those with "bad" family histories showed an avereage blood lipid (beta lipoprotein) level of 206 as against 193 for those with "good" family histories. This difference is significant, as is also the corresponding difference in the low density lipoprotein fraction sf 100-400. Moreover, both groups of drivers have higher lipid levels than the corresponding conductors. Obviously, the interactions of heredity and environment with serum lipoproteins and other factors are complicated,

and family studies are badly needed. Here is a most promising field for the exericse of Paul's "clinical epidemiology".[6]

Summing up, physically active workers seem to be healthier in middle age, which makes good sense from a phylogenetic point of view. In terms of ischaemic heart disease, the increasingly chairbound life may be a cause of its recent increase, may contribute to its occurrence in early middle age, may also be a factor in its severity. The indications are that in England such IHD as the habitually physically active do have, is milder and occurs later in life.[5,6]

Sedentary living is, of course, only part of the "epidemic constitution" of our time. Physically inactive men, like others, are often heavy smokers, and their work is particularly liable to involve them in nervous strain. Such men tend to be among the more prosperous, and are as overnourished as any.

The nervous factor. One of the puzzles — and challenges — in the international epidemiology of IHD is the very high rate in the United States as compared with Western Europe. Other countries also have high rates, for example, Australia and New Zealand; Finland is a law to itself, but the American experience has been studied most. The excess seems to be real and not a matter of diagnosis, etc. As yet there are few serious suggestions by way of explanation. Thus blood lipid levels do not seem to be very different in the United States, England and Sweden. Less is known about blood pressure, but there is nothing suggestive of gross international differences within the West. There might well be more obesity in the United States, but the relationships of obesity to IHD are not very clear or very strong. I fancy that on an average there is considerably less physical activity in America, certainly less than in Scandinavia; but again, the available evidence does not suggest that this would be a sufficient explanation for the observed differences in incidence of IHD. And it is the same with smoking. We are left with possible mental and emotional factors about which Osler was concerned even before the first World War. In the United States and Russia there is now great interest in possible relationships of disorder of higher nervous functions to IHD. American workers are strongly influenced by Psychonanalysis, the Russians by Pavlovian psychology. But the results seem much the same.

Rosenman and Friedman[18] describe a behaviour pattern characterized by an "intense, sustained drive for advancement . . . and chronic immersion in competitiveness and deadlines, both in vocation and in avocations". They write of "constant competitive striving" and "the pursuit of self-selected but rarely satisfying goals that lead to an incessant treadmill of work,

activities and deadlines . . . that require habitual acceleration of the pace of daily living". Such persons, they report, show hypercholesterolemia of perhaps 20 or 30 mgms. per cent, high Beta lipoprotein readings, premature arcus senilis, a higher norepinephrine secretion during work—and gross excess of IHD.

Now if this picture of outraged human nature, to borrow from Tawney, is true of any sizeable fraction of IHD victims, even those of middle-class, (and at present we do not know whether it is true or not), the observation would be exceedingly important. The descriptions correspond to the familiar picture of modern insecurity and status anxiety, to Fromm's market personality, and Riesman's other directed man of the times. As epidemiologists we must probe the relation of this behaviour to trends in modern society, to the emphasis on individual achievement; for example, the great social mobility, the trend to size and Organization, the profusion and confusion of roles, and many others. We must recognize the suggestion that coronary patients are casualities of the social system, indicated by the postulated relationships of IHD with sedentary occupation, as well as with the rising standard of living and over-nutrition. It is urgent that we in Public Health should seek systematically for evidence of such translation of social systems into morbid physiology and lethal pathology. If it is a fact that a major burden of disease arises from the successful functioning of our society, then the tasks confronting the "sanitary reformer" of today are as great as anything met by the 19th century pioneers of urban and industrial hygiene.

The emphasis on deadlines represents in malignant form the loss of the physiological, individual and more personal sense of timing characteristic of simpler societies which disappeared with the industrial revolution. Northrop, of Yale University, has written beautifully on this theme.[34] "When the cause of a disease is philosophical", he concludes, "the cure must also be philosophical". To which we might again reply in a small voice, "multiple casuality"—and proceed to

Diet. By now it is tolerably certain that fats in the diet affect blood lipid levels and that blood lipid levels affect coronary artheroma. However, it is also tolerably certain that more than fats in the diet affect blood lipid levels, more than blood lipid levels are involved in atheroma formation, and more than atheroma is needed for IHD. The triangular relationship between diet, blood lipids, and IHD is valid only in very general terms and at the extremes of human experience, comparing for example the wretched Bantu with Cape Town Europeans. Between, there is considerable uncertainty; and it is urgent, if sometimes difficult in the contemporary climate

of opinion, to keep an open mind and to seek more knowledge. The crudity, it may even be irrelevance, of available blood lipid fractions as indications of atherogenesis and thrombosis is only one of the major obstacles to progress.

At this point we can ask only two questions. How much is diet responsible for the modern increase of IHD in Western countries? And

TABLE 3. BRITISH DIET 1913-1957[15]

	Pounds of fat per person per year			
	1913	1938	1947	1957
Total fats	85	106	84	113
% E.F.A.*	7	7	7	7

* Essential fatty acids (taken as indicator of total polyunsaturated).

TABLE 4. MORTALITY FROM ISCHAEMIC HEART DISEASE IN RELATION TO FAT "CONSUMPTION"[12, 15, 17]

Country	*Fats* gms. per person per day*	*Ratio* $\frac{\text{Sat. F.A.}}{\text{Polyunsat. F.A.}}$	*Mortality from IHD***
U.S.A.	143	4.2	441
U.K.	139	6.0	241†
Sweden	131	4.2	147

* 1954-57.
** Males aged 50-54, 1957.
† England and Wales.

how much abatement of this epidemic may be expected from changes in the diet? There are puzzling discrepancies in the evidence.

In the United Kingdom, calorie consumption is higher by 13% since the first world war;[15] in view of the coincident reduction in physical activity this may be very important. Another epochal change is in the consumption of hardened fat. This was negligible before World War I; now about a third of the fats eaten in Britain have some hardened constituents.[18] However, the epidemiological evidence as illustrated in Table 3, gives no support to the current hypothesis on the crucial importance of *saturated* fat; there is nothing in the data to account for the steep and apparently steady increase of IHD in recent times. In our own studies among individuals (Fig. 2) we could find no correlation between fat intake and

blood lipid levels. The suggestion from both sources of data is that fat consumption levels are above the threshold that matters. To reduce blood lipids on the metabolic ward, the ratio of saturated to polyunsaturated fat in the diet needs to be 1:1 or even less, but the lowest ratio in Figure 2 is about 4:1.

Another piece of evidence which indicates that dietary fat is not the whole story is shown in Table 4. Differences in consumption of fats in the United

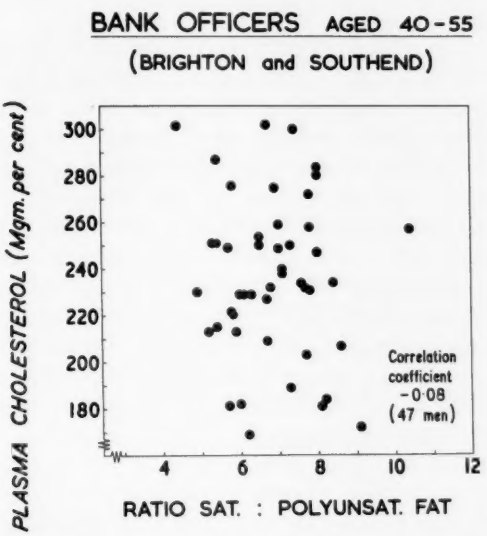


FIG. 2. Blood cholesterol in relation to diet. Each man weighed his food for two separate weeks.

States, United Kingdom, and Sweden fail to provide any sort of explanation of the differences in mortality from IHD in these countries. There are other inconsistencies when all Western countries for which data are available are included.

What are the prospects of controlling IHD by changing the diet to a ratio of unsaturated to saturated fats of 1:1? It is now clear that very high blood cholesterol levels can be reduced by this means by about one fifth and lesser levels by 15 or 10 per cent. The hope is that individuals whose diet is so changed will develop IHD at the lower rate of those who naturally have the lower cholesterol levels. On the basis of the Framingham study,[1] such lowering of lipids during middle age would still leave the incidence

of IHD in Americans higher than it presently is in England, and much higher than in Sweden. Perhaps this is starting altogether too late; we know there are many relevant changes earlier in life, well before middle age, both in blood lipids and in coronary arteries. Perhaps an attack on one cause, even if it proves to be the greatest, and sets the stage for the rest, is not enough. In any event, as already pointed out, American cholesterol levels alone do not account for the American excess of IHD.

THE NEXT STEPS

Be that as it may, what is urgently needed now are facts, not speculation. To judge from Figure 2 these facts will not be obtained from observation of what is "naturally" occurring in Western countries; there is too small a range and nearly everyone may be above the safety level in diet and in cholesterol level. Moreover, since multiple, powerful and widely diffused factors are involved, clear-cut answers are unlikely to emerge from further observation; and such studies in the West are yielding diminshing returns. Controlled experiments need to be made now, and the heroic proposals of my American colleagues for a mass trial of a diet high in polyunsaturated fat are indeed timely. Experiments on a grand scale must be carried out on more than diet; even now, it is known that mortality from IHD is substantially lower among men who have given up smoking. It may be possible to launch a trial in which cigarette smokers even if they do not give up their cigarettes change, at least, to cigars or a pipe. Planned increase of exercise in a large sedentary group will be more difficult to achieve, but we must begin to think about it; an hour's walking or its equivalent a day may be effective. Only with favourable results from such mass trials would we have much hope of conducting successful health education campaigns. More and more the challenge to Public Health seems to be to halt strong social trends, of which richer diets, heavy smoking, and less exercise are but the most obvious.

The scope for observation of "experiments of opportunity" remains great in certain population groups which are "developing" rapidly, such as those in West Africa or Israel. We may not have too much time to make such studies. I would regard them, too, as being most urgent.

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ROBERT W. QUINN*
ERNEST S. CAMPBELL**

Department of Preventive Medicine and Public Health, Vanderbilt University School of Medicine, Nashville, Tennessee

HEART DISEASE IN CHILDREN. A SURVEY OF SCHOOL CHILDREN IN NASHVILLE, TENNESSEE†

INTRODUCTION

Heart disease in children, and in particular, rheumatic heart disease, is not generally reported to public health departments; therefore, estimates of its prevalence in different geographic and climatologic areas, in specific age groups, in varying socioeconomic levels, and in other diverse environmental and familial conditions must be made on the basis of surveys. Prevalance surveys of heart disease have been conducted in some geographic divisions of the United States, and these surveys, along with clinical experience, are gradually adding to our knowledge of the prevalance of rheumatic fever and rheumatic heart disease. One of the earliest of these, in American Indian school children, was done by Paul.[1] Unfortunately, very few surveys of heart disease have been made in the southern states, too few, in fact to settle the question as to whether rheumatic heart disease is or is not less common in the southern United States when compared to the North. The hemolytic streptococcus, considered to be the agent factor in the etiology of rheumatic fever, has long been thought to be more frequently a cause of disease in children in the northern rather than southern states. However, recent studies of the prevalence of the hemolytic streptococcus have challenged this impression.[2,3]

The methods usually employed in determining the prevalence of heart disease, including questionnaires, health surveys, autopsy studies. hospital or clinic admissions, and actual examination of children, leave much to be desired. Probably the most commonly used method is that in which groups of children have been examined for evidence of heart disease, but even with this method, there are certain inherent inaccuracies which need

* Professor and Chairman, Department of Preventive Medicine and Public Health, Vanderbilt University, School of Medicine, Nashville 5, Tennessee. Formerly, Fellow and Instructor, Department of Preventive Medicine, Yale University School of Medicine, 1946-1949.

** Former Director, Heart Disease Control Program, Tennessee State Department of Public Health, U.S.P.H. Service. Present address: 803 Shades Crest Rd., Bluff Park, Birmingham 9, Alabama.

† Refers to the city of Nashville and its immediate suburbs.

to be taken into consideration in the interpretation of the findings. Individual differences in clinical acumen and experience, honest differences of opinion in the interpretation of clinical findings, the circumstances under which the examinations are done such as quietness and an adequate examining room and table, and the changing characteristics of murmurs in children from day to day and hour to hour (even organic murmurs associated with rheumatic heart disease change in children and young adults), are some of the factors constantly present to make a high degree of accuracy difficult to attain. The difficulties of quantitating the examiner in heart examinations are emphasized in a recent report by Feinstein.[4] We are acutely aware of the importance of the examiner in determining what rates will be found in a particular survey, and for this reason an attempt was made to study some of the variables due to the examiners. This part of the study will be reported later.

The present study was designed to augment our knowledge of the prevalence of rheumatic heart disease, rheumatic fever, and congenital heart disease in children living in a southeastern state, selected from urban and suburban environments, high, middle, and low socioeconomic groups, and from both white and negro races. An important stimulus for doing the survey was finding in this same area high carrier rates for hemolytic streptococci which are considered to be etiologic in rheumatic fever[5] The study served multiple purposes; as a case-finding measure, as an aid in the early diagnosis of heart disease and, equally important, it afforded an opportunity to study some epidemiologic factors significant in the etiology of rheumatic fever and rheumatic heart disease.

Nashville, Tennessee, the site of the study, is in Davidson County. In 1960 the estimated population of Nashville was 165,406 and for Davidson County 391,968. The 1957 estimate for total per cent of nonwhites living in Davidson County was 19.9. Inside the city limits the per cent of nonwhites was 38.7. Outside the city limits in Davidson County 3.7% were nonwhite. In Nashville and Davidson County, nonwhite means Negro in practically all cases. The city at an altitude of 606 feet is located on the Cumberland River and surrounded by low-lying hills, none of which reach an altitude of more than 1,160 feet, but which nevertheless afford some protection from winds. The climate, classified as subtropical, is not characterized by great extremes. The mean annual temperature for the 30 year period 1921-1950 was 60.1°F. with a mean maximum of 70.5°F. and a mean minimum of 49.7°F. Mean annual rainfull since 1910 has been 45.03 inches, occurring principally in the winter and spring with frequent thunder showers during the middle summer months. Nashville is located within the

Tennessee Valley Authority and has benefited greatly since its development.

METHODS

Selection of Schools and the Sample of Children to be Examined. The schools, located in Nashville and the immediate suburban areas, were classified as low, medium, or high, socioeconomically, based on 1950 census information. At the time the examinations were done, integration of the races in the school system had not yet involved the 6th and 7th grades. All the nonwhite children were placed in one group without differentiation as to socioeconomic level. The potential total number of children in the sample was 5,550. Four thousand and thirty-nine (4,039) children were examined. The reasons for this reduction in number were many, including absence on the day of examination, lack of parental consent, and loss of questionnaires by children, thereby cancelling participation. The details of the methods of selection of the schools and the sample of children to be examined are presented in the appendix and Table 6.

Pre-Examination Questionnaires. Questionnaires were sent home with each 12- and 13-year-old child to be filled out by one of the parents. Parental consent for examination was required and was indicated by the signature of the parent.

*Examination Methods.** On the day of examination, one or both of the physicians reported to the school and conducted examinations in the space provided, which was usually the school's clinic room. The public health nurse, with the help of the teachers, arranged for having the children ready for the examinations at the appointed time. The children undressed to the waist and examining gowns were furnished for the girls. Screens were usually used to afford privacy for the children and the examiner.

The actual examination consisted first in observing the child's general appearance, color, and state of nutrition. Examinations were conducted with the child in the upright and then in the supine position. The point of maximal impulse was determined digitally, followed by auscultation, first at the second right interspace at the right sternal border, then the left second interspace at the left sternal border, next at several different locations descending along the left sternal border, and finally at the apex. Transmission of murmurs was determined by listening at several locations progressing toward the left axilla, up into the neck, up and down the left sternal border starting from the second left interspace, and at other areas of the precordium, chest or back, when necessary. Auscultation at the second interspace and along the left sternal border was always done first, using the diaphragm of the stethoscope sometimes followed by the bell. At the apex both the diaphragm and bell were used.* Children with murmurs were always exercised to determine the effect of exertion on the murmur. Each examiner's findings were recorded on the back of the questionnaire

* Nomenclature and criteria for diagnosis outlined by the *New York Heart Association, 5th Ed.*, New York, 1953, were used as a guide.

* The stethoscopes used were the Rieger-Bowles type, manufactured by G. P. Pilling and Son. The diaphragms on this particular stethoscope are quite shallow and the bells used were truly bells, having deep hollowed-out bell sections rather than the shallow, dished-out type used on some stethoscopes.

immediately following the examination, using a check list and writing comments when necessary. Examinations were started in October 1957 and completed in May 1958.

RESULTS

The number of children examined is shown in Table 1, arranged according to the number of each sex, socioeconomic level, and race. There were 432 from high socioeconomic schools for white children, 1,464 from middle, 1,077 from low, and 1,066 from schools for negro children. The

TABLE 1. NUMBER OF CHILDREN EXAMINED BY SEX, AGE, AND SOCIOECONOMIC LEVEL

Socioeconomic level and race	*Total*	*Boys*	*Girls*	*Ages*			
				11 yr.	*12 yr.*	*13 yr.*	*14 yr.*
White:							
High	432	176	256	13	197	209	13
Middle	1,464	670	794	5	560	831	68
Low	1,077	495	582	19	447	558	53
Negro:	1,066	498	568	42	493	480	51
Total	4,039	1,839	2,200	79	1,697	2,078	185

total of 4,039 children was composed of 1,839 males and 2,200 females; 3,775 were 12 or 13 years of age, 79 were 11, and 185 were 14.

Clinical Impressions. The impressions recorded after examination are shown in Table 2, arranged by socioeconomic level, and Figure 1. A total of 103 children (2.5%) were classified as having rheumatic heart disease, while 31 (0.8%) were classified as having potential rheumatic heart disease, that is having either a history or rheumatic fever without valvular heart disease or a history of having inactive rheumatic fever. These children, plus 25 others who had rheumatic heart disease with a history of rheumatic fever, make a total of 53 children with a positive history of rheumatic fever. Possible rheumatic heart disease was the impression in 63 (1.6%). Congenital heart disease was thought to be present in 24 children (0.6%) and possible congenital heart disease in 121 (3.0%). The large number placed in the category of possible congenital heart disease reflects the unwillingness of the examiners to call some murmurs either organic or functional, and was an indication that these children needed further study. Unexplained murmurs, synonymous with so-called "functional" or "benign" or "physiologic" murmurs of childhood, were not considered to be associ-

ated with heart disease and were heard in 1,795 (44.4%) of the children. The total number of children classified as normal was 1,888 (46.7%).

The rates for rheumatic heart disease in each of the four socioeconomic categories were: high 1.6%, middle 1.6%, low 4.5%, and negro 2.2 per cent. The rate for the negro children, although higher than that for the high and middle groups, was not significantly higher statistically and was lower than the rate of 2.8% for all white children combined. The rate of

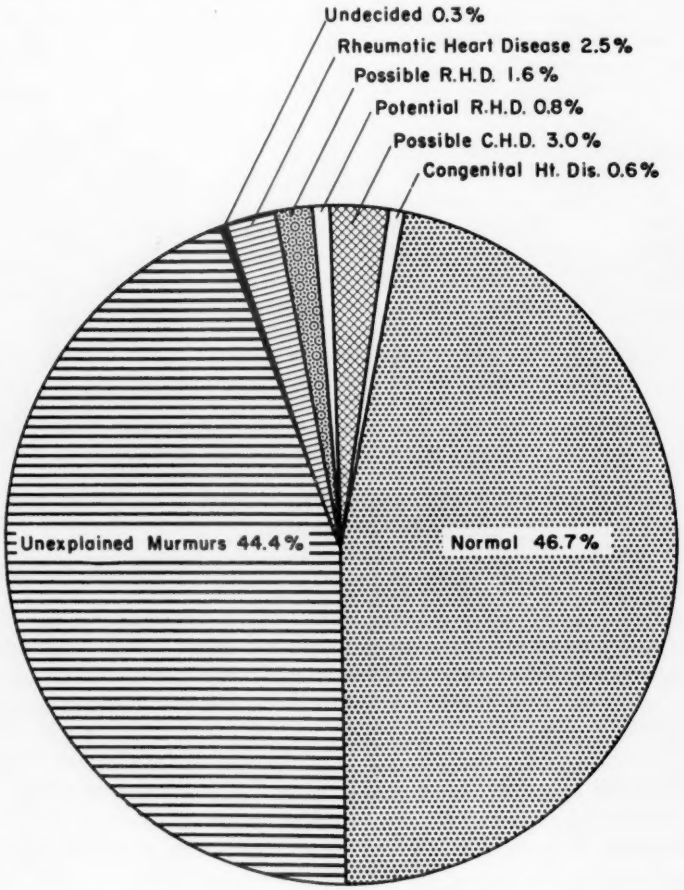

Figure 1. RESULTS OF EXAMINATIONS OF 4039 SCHOOL CHILDREN

4.5% with rheumatic heart disease in the low socioeconomic group was significantly higher than the rates for any of the other groups ($P<.001$).

Table 3 and Figure 2 show the distribution by impression of the 103 with rheumatic heart disease. The most frequent impression was mitral insufficiency found in 60 children (58.3%). Mitral stenosis was the next

TABLE 2. RESULTS OF EXAMINATION OF 4,039 SCHOOL CHILDREN

Impression	*Socioeconomic level and race*									
	White									
	High		*Middle*		*Low*		*Negro*		*Total*	
	No.	*%*	*No.*	*%*	*No.*	*%*	*No.*	*%*	*No.*	*%*
Normal	152	35.2	676	46.2	581	53.9	479	44.9	1,888	46.7
Unexplained murmurs	237	54.9	688	47.0	374	34.7	496	46.5	1,795	44.4
CHD*	2	0.5	9	0.6	8	0.7	5	0.5	24	0.6
CHD possible	16	3.7	42	2.9	32	3.0	31	2.9	121	3.0
RHD potential	1	0.2	8	0.5	11	1.0	11	1.0	31‡	0.8
RHD possible	14	3.2	15	1.0	17	1.6	17	1.6	63	1.6
RHD**	7	1.6	24	1.6	49	4.5	23	2.2	103	2.5
Undecided†	3	0.7	2	0.1	5	0.5	4	0.4	14	0.3
Total	432	100%	1,464	100%	1,077	100%	1,066	100%	4,039	100%

* CHD—Congenital heart disease.

** RHD—Rheumatic heart disease.

† Undecided—It was not possible for the examiners to classify these 14 under a single impression. They include 3 with congenital or possible congenital heart disease; 6 with possible rheumatic heart disease; 6 with possible rheumatic or possible congenital heart disease; 3 with rheumatic or congenital heart disease; 1 with either an unexplained murmur or possible congenital heart disease; and 1 with possible congenital heart disease or rheumatic heart disease.

‡ This is the number of children considered to have only potential rheumatic heart disease after obtaining further information. Potential rheumatic heart disease is a diagnostic term equivalent to a history of rheumatic fever without evidence of rheumatic heart disease, or to inactive rheumatic fever.

most frequent impression, in 21 (20.4%); and aortic insufficiency, the least frequent of isolated valvular lesions was thought to be present in 2 children (1.9%). Combined valvular lesions were present in the form of mitral insufficiency and stenosis in 14 (13.6%), mitral insufficiency, mitral stenosis, and aortic insufficiency in 4 (3.9%).

History of Rheumatic Fever, Heart Trouble, or St. Vitus' Dance. The information obtained from questionnaires sent home with the child and answered by a parent indicates a history of rheumatic fever in 65 (1.6%) of all children examined, in 1.4% of high socioeconomic children, 0.9% of

the middle, 2.0% of the low, and 2.4% of the negro children. Histories obtained from questionnaires are open to question, and after rechecking, it was determined that only 53 children had bona fide, acceptable histories of rheumatic fever. Fifty-one children were reported by their parents as

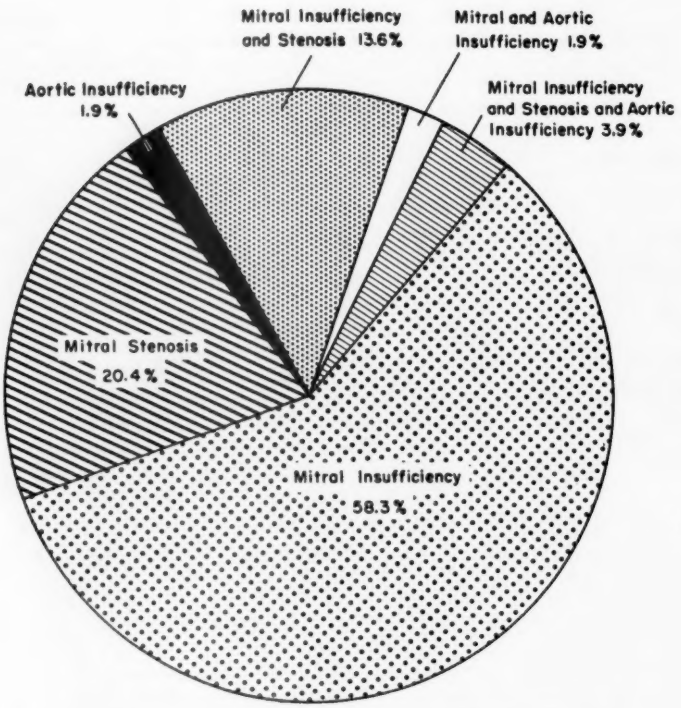
Mitral Insufficiency and Stenosis 13.6%
Mitral and Aortic Insufficiency 1.9%
Aortic Insufficiency 1.9%
Mitral Insufficiency and Stenosis and Aortic Insufficiency 3.9%
Mitral Stenosis 20.4%
Mitral Insufficiency 58.3%

Figure 2. CLINICAL IMPRESSIONS FOR 103 CHILDREN CONSIDERED TO HAVE RHEUMATIC HEART DISEASE.

having heart trouble, but after examination none of these was considered to have heart disease. The opposite was observed too, that is, the presence of rheumatic and congenital heart disease with no knowledge by the parents. Only 19 (18.4%) of the parents of the 103 children with rheumatic heart disease and 8 of the parents of the 24 children with congenital neart disease knew of the condition.

Relative with a History of Rheumatic Fever. The data in Table 4, also obtained from questionnaires, show for each socioeconomic group the

average number of children per family, and the per cent of parents, siblings, aunts or uncles, and of first cousins with a history of rheumatic fever. The parents of the high and middle groups had histories of rheumatic fever approximately twice as frequently as parents in the low and negro groups. This is the opposite of what was found from histories of the children, where the low socioeconomic and negro children had histories of rheumatic fever approximately twice as often as the high and middle groups. The per cent of siblings of the examined child with a history of rheumatic fever was 3.9% for the high group, 2.9% for the middle, 5.8% for the low, and 3.8% for the negro. The per cent of aunts or uncles, and of first cousins of

TABLE 3. CLINICAL IMPRESSIONS FOR 103 CHILDREN CONSIDERED TO HAVE HEART DISEASE

*M.I.**		*M.S.***		*A.I.*†		*M.I. and M.S.*		*M.I. and A.I.*		*M.I., M.S. and A.I.*		*Total no.*
No.	*%‡*	*No.*	*%*	*No.*	*%*	*No.*	*%*	*No.*	*%*	*No.*	*%*	*with RHD*
60	58.3	21	20.4	2	1.9	14	13.6	2	1.9	4	3.9	103

* M.I.—Mitral insufficiency.
** M.S.—Mitral Stenosis.
† A.I.—Aortic insufficiency.
‡ %—Means per cent of total with rheumatic heart disease (RHD).

the examined child with a history of rheumatic fever, increased gradually from the high to the middle to the low socioeconomic groups but consistently was much lower in the negro families.

Rheumatic Fever in Children from Families Having Parents with and without a History of Rheumatic Fever. Table 5 is arranged to show the family history of rheumatic fever, indicating whether it was present in both parents, a single parent, or neither parent. With the exception of the children examined, in whom there was no rheumatic fever by history, significantly more of the siblings of the examined child and of aunts, uncles or first cousins in families in which both parents had rheumatic fever, had histories of rheumatic fever than was the case in families in which a single parent or neither parent had a positive history. The children examined who had one rheumatic parent also had significantly more relatives with a history of rheumatic fever among their siblings, aunts, uncles, or first cousins than did those children of whom neither parent was rheumatic.

Socioeconomic Level and Race. Rheumatic heart disease was found in 1.6% of the children examined in both the high and middle socioeconomic

levels, 4.5% of the children in the low socioeconomic level, and 2.2% of the negro children. Potential rheumatic heart disease (inactive rheumatic fever without rheumatic heart disease) was present in 0.2% of the high, 0.5% of the middle, and 1.0% of both low socioeconomic white children and negro children. Congenital heart disease was present in about equal proportions in each of the socioeconomic groups and races, 0.5 in the high, 0.6 in the middle, 0.7 in the low, and 0.5 in the Negro.

Crowding in the Home. Crowding increased significantly from homes in high socioeconomic areas to homes in lower socioeconomic areas when

TABLE 4. HISTORY OF RHEUMATIC FEVER IN PARENTS, SIBLINGS, AND RELATIVES OF THE EXAMINED CHILD

Socioeconomic level	*No. of children*	*Average no.* of children in family*	*% of parents with history of R.F.*	*% of siblings with hisotry of R.F.*	*% of aunts or uncles with history of R.F.*	*% of first cousins with history of R.F.*	*% of all relatives with history of R.F.*
White:							
High	432	1.57	1.9	3.9	5.1	5.3	5.2
Middle	1,464	2.10	2.0	2.9	6.1	7.1	6.6
Low	1,077	2.30	1.0	5.8	7.2	7.2	7.2
Negro:	1,066	3.44	1.1	3.8	2.2	1.8	2.0
Total	4,039	2.70	1.6	4.0	5.2	5.5	5.2

* Estimated from the number of persons in the household minus two parents minus the index child.

measured according to the number of persons per room, the number of persons in the child's bedroom or sleeping quarters, and the number sleeping in the child's bed. Ninety-two per cent (92%) of the homes of the high socioeconomic group had less than one person per room. This decreased to 32% in the homes of negro children. There were twice as many persons sleeping in the examined child's quarters when the negro children were compared with children of high socioeconomic level. From the data, 10% of the children of the high socioeconomic group had two or more persons sleeping in the same bed, while 42% of the negro children had two or more bed mates. The middle and low socioeconomic groups were intermediate between the negro and high socioeconomic groups in regard to crowding in the home, the low socioeconomic homes being only slightly less crowded

than the negro homes. Both negro and low socio-economic white homes were consistently more crowded when compared with averages for the four socioeconomic areas. White children in the low socioeconomic group came from homes which were much more crowded than the middle or high groups and they had significantly more rheumatic heart disease. The negro children's homes were even more crowded than the low group white homes, yet the rheumatic heart disease rates were lower than for the white children as a group.

TABLE 5. HISTORY OF RHEUMATIC FEVER IN CHILDREN OF FAMILIES WITH PARENTS WITH AND WITHOUT A HISTORY OF RHEUMATIC FEVER

History of rheumatic fever	*No. of families*	*No. and % of children examined with R.F.*		*No. and % of families with siblings of examined child with R.F.*		*Aunts, uncles or first cousins in family with R.F.*	
		No.	*%*	*No.*	*%*	*No.*	*%*
Both parents	5	0	0.0	4	80.0	4	80.0
One parent	120	6	5.0	6	5.0	20	16.6
Neither parent	3,914	47	1.3	152	3.8	380	9.7
Total	4,039	53	1.4	162	4.0	404	10.0

DISCUSSION

The results indicate that rheumatic heart disease is as common in the children examined in this middle Tennessee area as in any other section studied in the United States with the one exception of some industrial cities in Connecticut where a rate of 6% was found.[5] The few heart disease surveys done in the United States during the past two decades have given rheumatic heart disease rates ranging from the high of 6% in industrial towns in Connecticut to a low of 0.32% in a warm, dry, residential town in California.[6] A survey in Pensacola, Florida, showed a rate of 0.38%[7] and another in Dade County, Florida, a rate of 0.50%.[8] One of the authors had previously conducted surveys in three other areas of the United States, and the results of these surveys are probably more comparable to those of the present study since the same examiner would tend to minimize the variables introduced by several examiners. He found the Nashville prevalence rate for rheumatic heart disease (2.5%) to be slightly higher than that found in urban areas in Connecticut (2.45%),[5] higher than that in Madison, Wisconsin (2.2%)[9] and considerably higher than that in Dublin, Georgia

(1%).[10] The rates obtained by each examiner in the present survey were recorded and analyzed separately. It is noteworthy that differences between the two were not great enough to invalidate the general impressions stated above in regard to comparative rates.

The history of rheumatic fever obtained for 1.6% of the children examined is slightly lower than that obtained from Connecticut children (1.9%) living in urban areas.[11] Taking into consideration possible errors in histories, these two figures, 1.6% in Nashville and 1.9% in certain urban areas in Connecticut, are not much different. Only a fourth of the children with rheumatic heart disease in this study had a history of rheumatic fever, but more negro children reported a history of rheumatic fever than were found to have rheumatic heart disease. Do these findings suggest that the negro child in Nashville is less likely to acquire rheumatic fever than the white child, but that when he does get it, he is more likely to acquire rheumatic heart disease? Much more information is necessary to confirm this suspicion.

When physicians who conduct surveys such as this one find high prevalence rates, they may be disturbed because of their inability to prove their observation or dispel the doubts of others. It is reassuring, for the surveyors at least, to obtain high rates from other sources. The large percentage of families in each of the socioeconomic groups reporting rheumatic fever in parents, aunts, uncles, or cousins (5.2%), is evidence that the high rates of rheumatic heart disease found by the examiners may be a fairly accurate picture of the rheumatic fever and rheumatic heart disease problem in the community. The per cent of Nashville white children reporting rheumatic fever in relatives is actually higher than the per cent of urban Connecticut school children with a family history of rheumatic fever or rheumatic heart disease in the survey done in Connecticut 12 years ago.[5] The high percentage of families (4%) for all groups, who reported rheumatic fever in siblings of the child examined, is further evidence that rheumatic fever is fairly common in Nashville. One other bit of information which might support the finding of high rates of rheumatic heart disease is the data from long-term studies of the frequent occurrence of the hemolytic streptococcus in the throats of Nashville school children.[12] If these streptococci are capable of an etiologic role in the occurrence of rheumatic fever, the rheumatic potential is great for these organisms are certainly abundant.

The data in Table 5 indicate a high familial prevalence of rheumatic fever in Nashville families in which one or more parent had rheumatic fever. However, 1.4% of the children from families in which neither parent gave a positive history were found to have had rheumatic fever.

Actually, 4 per cent of the siblings and 10% of aunts, uncles or cousins in these families also had histories of rheumatic fever. Obviously, such data as these are not particularly informative in terms of genetic inheritance. Furthermore, the knowledge of and reporting of rheumatic fever by questionnaire varies considerably in families and would tend to introduce inaccuracies difficult to control.

The rates for rheumatic heart disease in each socioeconomic group are in keeping with what might be expected, that this disease is found more frequently in people of the lower socioeconomic classes where living conditions are substandard and more crowded. In this regard, crowding in the home was associated with significantly higher rheumatic heart disease rates in the low socioeconomic white children. The degree of crowding in the homes of negro children was even higher than that in the low socioeconomic children, but the rheumatic heart disease rates were lower. Crowding seemed to be uniformly associated with higher rates in the white children, but for negro children the effect of crowding was not nearly so marked. These findings suggest the question: "Is there a lower degree of susceptibility to rheumatic fever in the Negro?"

The results of various surveys in regard to the prevalence of rheumatic fever and rheumatic heart disease in negro children compared with white children are not consistent. Wolff's[13] study of childhood mortality from rheumatic fever indicated higher mortality from rheumatic fever and rheumatic heart disease in nonwhite compared with white children. These findings were commented on by Paul, who wrote: "The picture of rheumatic fever and rheumatic heart disease in negroes in the United States today is a more serious one than in whites.[14] Collins' conclusions[15] were the same as Wolff's, namely that mortality rates for active rheumatic fever and chronic rheumatic heart disease are consistently higher for the negro than for white children 5 to 19 years of age. The incidence of active rheumatic fever at the time of the morbidity survey,[15] 1935-36, was about the same for white and Negro. A survey by Saslaw and others[8] revealed no rheumatic heart disease among white children, all five cases being in negro children. In Nashville, at the time of the present survey, rheumatic heart disease would appear to be considerably less frequent in negro children (2.2%) than in white children of the low socioeconomic group (4.5%) and slightly less common than in white children of all the socioeconomic groups combined (2.8%).

The finding of congenital heart disease in about equal proportions in each of the socioeconomic groups and races and of rates remarkably similar to those in Georgia,[10] Connecticut,[5] and Wisconsin,[9] where they were 0.5% ±

0.1% suggests that etiologic and epidemiologic factors important in congenital heart disease may be common to each of these geographical areas and to each of the races. The per cent of children found to have so-called unexplained or functional or physiologic murmurs (44.4%) is not surprising. As a matter of fact, it is slightly lower than that reported in many surveys.

SUMMARY AND CONCLUSIONS

1. The results of a survey of heart disease among 4,039 twelve and thirteen year old white children selected from high, middle, and low socioeconomic levels and negro children living in Nashville, Tennessee, as well as its immediate suburbs, are presented in this paper.

2. The cardiac examinations were interpreted as follows: 2.5% had rheumatic heart disease, 0.8% had potential rheumatic heart disease; 1.6% possible rheumatic heart disease; 0.6% congenital heart disease; 3.0% possible congenital heart disease; 44.4% had unexplained or "functional" murmurs; and 46.7% were normal.

3. The frequency of clinical impressions for the 103 children with rheumatic heart disease were: mitral insufficiency, 58.3%; mitral stenosis, 20.4%; aortic insufficiency, 1.9%; mitral insufficiency and stenosis, 13.6%; mitral and aortic insufficiency, 1.9%; and mitral and aortic insufficiency and mitral stenosis, 3.9%.

4. The rates of rheumatic heart disease by socioeconomic groups and race were: high 1.6%, middle 1.6%, low 4.5%, and Negro 2.2%. The rate for negro children was lower than the combined rate for all white children (2.8%).

5. A history of rheumatic fever was obtained for 1.6% of all children, 1.4% for children in the high socioeconomic level, 0.9% in the middle, 2.0% in the low, and 2.4% for the negro children.

6. Crowding in the home and other environmental characteristics which accompany low socioeconomic level were associated with higher rheumatic heart disease rates in the white children. The effect of crowding and low socioeconomic level was less marked in negro children. These children, who, as a group were in the low socioeconomic level, and whose homes were the most crowded, had slightly lower rates than the white children.

7. The per cent of relatives (aunts, uncles, or first cousins) of the examined children who had rheumatic fever increased gradually from the high to the middle and from the middle to the low socioeconomic children. Only 2% of the relatives of negro children had a history of rheumatic fever. This was much lower than for white children.

8. There was a high familial prevalence of rheumatic fever by history, highest in families in which both parents were rheumatic, next highest in families with one rheumatic parent, and least frequent in families in which neither parent had rheumatic fever.

9. The results indicate that rheumatic heart disease is as common in the children examined in this middle Tennessee area as in any other section of the United States which has been studied.

TABLE 6. NUMBERS IN SAMPLE AND NUMBERS EXAMINED

Socio-economic group and race	*Estimated number in 6th and 7th grades*	*Available 12 and 13 year olds**	*No. in sample selected*	*No. of sample examined*	*Per cent of sample examined*
White:					
High	1,150	1,162	1,150	432(705)**	37.6(60.4)**
Middle	2,800	2,354	1,400	1,464	100+
Low	1,900	1,295	1,200	1,077	89.7
Negro:	2,000	1,297	1,300	1,066	82
Total	7,850	6,108	5,050	4,039	79.9

* These are the numbers of 12 and 13-year-old children enrolled at the beginning of the fall term in 1957.

** Seven hundred and five (60.4%) of the sample selected from high socioeconomic schools actually were examined. The results of 273 of these examinations, performed by a third examiner, were eliminated because it was not possible for either of the two other examiners to check his findings. The two authors were able to consult each other frequently to check histories and clinical impressions.

10. Some of the epidemiologic factors thought to play a role in the relatively high rheumatic heart disease rates were crowding in the home and low socioeconomic status for the white children at least (the effect of crowding and low socioeconomic status was less marked for negro children), the high frequency of family histories of rheumatic fever for the white children (family histories of rheumatic fever were much less frequent in negro families), and possibly the frequent occurrence of hemolytic streptococci in the throats of Nashville school children.

APPENDIX

Selection of Schools and the Sample of Children to be Examined (Table 6.)

Only schools within the tract area were considered for selection because it was possible to obtain socioeconomic characteristics for these areas. The characteristics used to classify schools serving low socioeconomic areas were i) monthly rental from

$10 to $34, value of the dwelling $2,000 to $5,999, ii) family income $1,000 to $3,999 yearly, and iii) percent of houses with more than one person per room 17.5 to 32.5. Schools placed in the middle socioeconomic groups served areas with i) median monthly rental from $30 to $54, value of dwellings $6,000 to $10,999, ii) median family income $2,000 to $4,999, and iii) 25 to 17.5% of homes with more than one person per room. Criteria for schools serving high socioeconomic areas were i) median monthly rental from $60 a month up, value of homes owned $12,000 and up, ii) median annual income $5,000 and up, and iii) per cent of homes with more than one person per room from 0 to 2.5. Results of classification of the schools in this manner agreed with the Nashville and Davidson County Boards of Education appraisals of the socioeconomic areas which the schools served.

The total enrollment for the Nashville and Davidson County Schools on 6 September 1956 was obtained. From the school enrollment in the 5th and 6th grades at that time, an estimate was made of the number of children expected to be in the 6th and 7th grades during the school year of 1957. Based on the age distribution of children in the schools, it was calculated that the 6th and 7th grades would yield the largest number of children 12 and 13 years of age. This age group was selected because these children had passed the age at which rheumatic fever most often occurs for the first time, that is, sometime between the ages of 7 through 10, and moreover, there would have been time for them to develop murmurs characteristic of rheumatic heart disease by the time they were 12 or thirteen.

The total numbers, not necessarily 12 or 13 years of age, which would be enrolled in Nashville and Davidson County Schools within the tract area in the 6th and 7th grades in 1957, were estimated to be 1,900 in the schools serving low socioeconomic areas, 2,800 in the middle, 1,150 in the high, and 2,000 Negro. Accordingly, the 1,900 children in schools serving low socioeconomic areas were randomly placed in three clusters of 600 to 650 and two clusters were selected randomly, giving about 1,200 children to be examined. The 2,800 children in the schools serving middle socioeconomic areas were divided randomly into four clusters of about 700 each and two clusters were selected, giving approximately 1,400 children to be examined. There were only two schools within the city limits which could qualify as serving a high socioeconomic area, with 150 children. Most of the schools serving high socioeconomic areas were in the suburbs of Nashville, outside the city limits in Davidson County. The estimated total number was 1,150, so all these schools were included in the sample, in order to obtain an adequate number from this socioeconomic level. It was estimated that about 2,000 negro children would enroll in the 6th and 7th grades in 1957. Since it was known that the number of 12 and 13 year old children in the 6th and 7th grades would be smaller percentage-wise than in the schools serving white children, it was decided to select all 10 Nashville negro schools having 6th and 7th grade negro children and one large Davidson County negro school in order to secure adequate numbers. The total number of 6th and 7th grade negro children in these schools who would be 12 or 13 years of age was estimated to be about 1,300.

The records for 220 children who had returned consent and history forms could not be used either because the history was not completed, or the physical examination was not done. This was the year of the Asian influenza epidemic and many children were absent from school on the days of examination. Forty-nine of these unused records were from children in the high socioeconomic group, 113 from the middle, 14 from

the low, and 44 from negro children. The reasons why a relatively small percentage of children in high socioeconomic schools participated in the study are not clear. Without having the facts at hand, it would seem useless to speculate on the reasons or to speculate on whether the characteristics of the group which did not participate were different as far as the presence or absence of heart disease is concerned.

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CHARLES H. RAMMELKAMP, JR.*
BERTRAND L. STOLZER**

Streptococcal Disease Laboratory, Francis E. Warren Air Force Base, Wyoming, and the Departments of Preventive Medicine and Medicine, Western Reserve University School of Medicine, Cleveland, Ohio

THE LATENT PERIOD BEFORE THE ONSET OF ACUTE RHEUMATIC FEVER†

INTRODUCTION

John R. Paul has been interested in the latent period and the incubation period of a variety of infectious diseases because careful study of the host during these periods frequently yields important clues concerning pathogenesis. In the case of rheumatic fever, the latent period begins at the onset of symptoms of the acute group A streptococcal respiratory infection and ends at the time of development of symptoms of acute rheumatic fever. Thus, in the early phase of the latent period, the patient exhibits signs and symptoms of tonsillitis or pharyngitis, whereas in the second phase, unless a suppurative complication supervenes, the patient is asymptomatic.[1]

The occurrence of a latent period prior to the development of symptoms of acute rheumatic fever or acute glomerulonephritis is responsible for the suggestion that these two complications of group A streptococcal infections represent some form of altered tissue reactivity of an immunological nature.[2,3] Ranz, Boisvert, and Spink[4] studied a group of patients during the latent period and suggested, on the basis of bacteriologic studies, that reinfection with a new type of streptococcus may be necessary for the development of these non-suppurative complications. In order to define the normal latent period as well as to determine the effect of reinfection during this time interval the experience at the Streptococcal Disease Laboratory was reviewed. At this facility, all patients with clinically evident streptococcal disease or acute rheumatic fever were hospitalized and studied by standard methods including bacteriologic and serologic techniques.

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*Professor of Medicine and Associate Professor of Preventive Medicine, Western Reserve University School of Medicine.
**Clinical Instructor in Medicine, University of Pittsburgh School of Medicine.
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Thus, it was possible to define the latent period and to examine some of the factors which might be expected to alter its length.

METHODS

The records of all airmen admitted to the hospital at Francis E. Warren Air Force Base between January 1949 and June 1953 with symptoms of acute rheumatic fever were studied. A final diagnosis of acute rheumatic fever was made utilizing the criteria of Jones[6] with slight modification.[9]

During the same period that the patients with acute rheumatic fever were observed, all airmen with symptoms of an acute respiratory illness associated with either fever or exudative tonsillitis or pharyngitis were admitted to a special study unit. After a history, physical examination, oropharyngeal culture and an acute phase serum specimen were obtained, half of the patients received therapy with an antibiotic and the remaining patients were given symptomatic therapy and served as controls. These studies were designed to determine the effectiveness of various thrapeutic regimens in the prevention of acute rheumatic fever. As a part of these investigations each patient was carefully observed during convalescence to determine whether complications developed. A second culture of the oropharynx and a specimen of serum were obtained approximately three weeks after the onset of the streptococcal illness. If the patient developed rheumatic fever, additional cultures were taken. In each case an effort was made to date accurately the onset of symptoms of the streptococcal infection and the time the first rheumatic symptoms appeared. The period between these two events was calculated to the nearest day.

TABLE 1. PATIENTS WITH ACUTE RHEUMATIC FEVER INCLUDED IN STUDY

Category	*Number*	*Per cent*
Acute rheumatic fever	565	100
Observed during preceding infection	274	48
Date of onset of rheumatic fever not known	23	4
Total analyzed	251	44

The oropharyngeal swabs were rubbed on sheep blood agar plates and after 18-24 hours incubation *beta* hemolytic colonies were isolated in pure culture. Classification of these cultures into serologic groups and types was performed by standard procedures.[7] Antistreptolysin 0 titers were determined at the same time on all sera collected from any one patient.

RESULTS

During the period of this study 565 patients exhibiting the classical features of acute rheumatic fever were observed on the hospital wards (Table 1). Of these patients, 274 or 48 per cent had been hospitalized previously because of an acute respiratory illness. Examination of these

274 records showed that in 23 the dates of onset of the symptoms of acute rheumatic fever were uncertain, so that the present analysis was limited to the remaining 251 patients.

Table 2 presents the latent periods of these 251 patients grouped in intervals of three days. Since prior studies had demonstrated that indi-

TABLE 2. THE LATENT PERIOD OF 251 PATIENTS WITH RHEUMATIC FEVER OBSERVED DURING A PRECEDING RESPIRATORY INFECTION

	Initial culture				
	Group A streptococcus				
Latent period (days)	*Single infection†*	*Two infections‡*	*Received therapy*	*Negative**	*Totals*
0-2	2	0	5	6	13
3-5	3	0	2	0	5
6-8	6	0	4	1	11
9-11	10	1	2	1	14
12-14	16	1	2	3	22
15-17	11	1	4	4	20
18-20	18	2	2	3	25
21-23	15	2	3	1	21
24-26	17	4	2	4	27
27-29	6	3	2	4	15
30-32	5	2	1	1	9
33-35	4	1	1	2	8
36-45	11	15	6	10	42
Over 45	3	6	6	4	19
Totals	127	38	42	44	251

† Convalescent culture was negative or showed the same type isolated on initial culture.

‡ A type different than that isolated on the initial culture was identified during convalescence. Nine of these patients received antibiotics of whom 7 exhibited an interval of over 35 days.

* Eighteen of these patients subsequently showed a positive culture.

viduals exhibiting long latent periods usually had experienced an intervening streptococcal infection as determined by bacteriologic, serologic or historical evidence[6,8] such patients were listed together. Of the entire group of 251 patients 61 or 24 per cent showed latent periods of over 35 days, and 19 or 8 per cent of the intervals were over 45 days. Based on bacteriologic studies alone, 21 of these 61 patients with long latent periods experienced a reinfection, whereas reinfections were demonstrated

in only 17 of the 190 patients with latent periods of less than 36 days. In addition, 26 of the patients with long latent periods either showed no group A streptococci on admission to the hospital for the observed respiratory illness or they received therapy with an antibiotic. In both instances, it appeared reasonable to assume that either an inapparent or a continuing infection with a group A streptococcus probably occurred after the observed illness and before the onset of rheumatic fever.[9] For these reasons it appeared unlikely that most of the cases of acute rheumatic fever with latent periods of over 35 days were directly precipitated by the observed respiratory illness.

TABLE 3. CHARACTERISTICS OF THE PRECEDING RESPIRATORY ILLNESS IN 127 PATIENTS WHO RECEIVED NO SPECIFIC THERAPY AND WHO SHOWED NO BACTERIOLOGIC EVIDENCE OF REINFECTION PRIOR TO ONSET OF RHEUMATIC FEVER

	Number		*Per cent present*
	Observed	*Present*	
Exudate	127	98	78
Increase in antistreptolysin titer	118	109	92
Leukocytosis of 12,000 or greater	119	109	92

Since these studies were conducted in a population experiencing epidemics of streptococcal and non-streptococcal respiratory diseases, it was essential to establish the etiology of the observed respiratory illnesses. In addition, it was assumed that specific antistreptococcal therapy of the observed illness or the occurrence of a new, inapparent, streptococcal infection prior to the onset of rheumatic symptoms might affect the length of the latent period. Thus, the 251 patients seleced for analysis were subdivided into the various groups presented in Table 2.

There were 127 patients who showed group A streptococci from the culture obtained on hospitalization, who received no specific therapy with antibiotics or sulfonamide drugs, and whose convalescent cultures were either negative or showed the infetcing type of streptococcus. The data presented in Table 3 document the fact that the observed respiratory illnesses in these patients were caused by the streptococcus. The majority of the patients was found to have an exudative lesion of the pharynx or tonsils and elevation of the total leukocyte count. As many as 92 per cent exhibited a significant increase in the titer of antistreptolysin during convalescence.

The distribution of the latent periods observed in these 127 patients is shown in Figure 1 as well as in Table 2. In this group the latent period was greater than 35 days in only 14 or 11 per cent. In 3 patients the latent period was over 45 days. Although bacteriologic evidence of re-infection was lacking in these 14 patients, it seems probable that most had experienced a new, unobserved infection which was not detected by the bacteriologic technique employed. In contrast, the majority of the re-

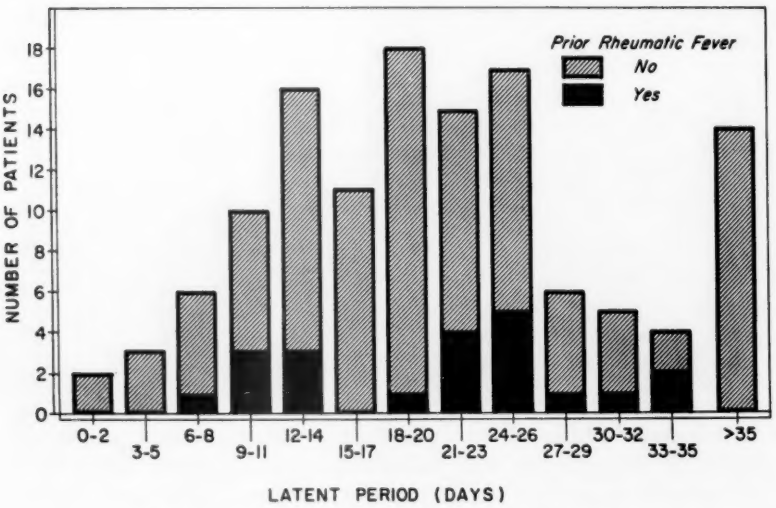

FIG. 1. The latent period in acute rheumatic fever.

maining 113 observed illnesses probably represent infection by a single serologic type.

The mean interval for those patients exhibiting a latent period of less than 36 days was 18.6 days; the median was 19 days. The frequency distribution of the latent periods roughly parallels a normal distribution curve. This is especially true if those patients who have experienced a prior attack of rheumatic fever, as depicted in Figure 1, are eliminated from consideration. The 21 individuals who were experiencing a recurrent attack of rheumatic fever did not exhibit a shortened latent period, nor did any show a latent period of over 35 days.

Of the 113 patients with latent periods of less than 36 days, 11 or 10 per cent, developed rheumatic symptoms during the first 8 days. There were 2 patients whose symptoms of acute rheumatic fever appeared to

develop near the time of onset of the acute respiratory illness. In one the initial antistreptolysin titer was 500 indicating that this patient had recently experienced another streptococcal infection which may have accounted for the apparent short latent period. However, he had no recollection of a recent sore throat prior to the observed illness and examination showed no exudate on the tonsils or pharynx. It is possible that the symptom, sore throat, was associated with the onset of acute rheumatic

TABLE 4. AVERAGE LATENT PERIOD ACCORDING TO THE TYPE OF GROUP A STREPTOCOCCUS CAUSING THE INFECTION

Type of streptococcus	*Average interval (days)*	*Number of patients**
14	19	52
5	20	19
30	17	10
19	17	8
24	15	6
18	21	5
NT	22	5
1	13	3
3	12	3
6	21	1
4	24	1

* Limited to the 113 patients who received no therapy and showed no evidence of reinfection. The 14 patients with intervals greater than 35 days are omitted.

fever and did not represent a new respiratory illness. The second patient exhibited exudative pharyngitis and the initial antistreptolysin titer was 125 which later increased to 250 units. He gave no history of a prior respiratory illness. Thus, in this patient the rheumatic symptoms appeared without a latent period.

The relationship of the length of the latent period to the type of infecting organism is presented in Table 4. There were 10 different types causing the initial infection in addition to the 5 patients from whom a non-typable strain was isolated. The majority of infections was due to type 14. The relative frequency of the types isolated paralleled the frequency with which these types were present in other patients with strptococcal pharyngitis who did not develop rheumatic fever. The length of the latent period did not appear to be a function of any particular type.

The relationship of the latent period to the titer of antistreptolysin in the acute phase sera as well as to the average increase in titer demon-

strated during convalescence is shown in Table 5. No definite conclusion can be made regarding the average initial titers or the average increase in titers in those patients with short or long latent periods. It is possible that the slightly higher average initial titers exhibited by the 5 patients in the 0-5 day interval is due to the fact that these patients had already experienced an increase in antibody at the time the initial, acute phase sera were obtained. This suggestion is confirmed by the relatively small in-

TABLE 5. THE RELATIONSHIP OF THE LATENT PERIOD TO ANTISTREPTOLYSIN TITERS

Latent period (days)	*Number of patients**	*Average antistreptolysin titer*	
		Initial	*Increase at 3 weeks*
0-2	2	313	125
3-5	3	187	51
6-8	5	99	336
9-11	8	137	492
12-14	16	173	332
15-17	11	100	256
18-20	18	109	409
21-23	14	136	240
24-26	16	119	208
27-29	5	142	228
30-32	5	175	233
33-35	4	96	263
Greater than 35	11	150	471

* Includes only those patients with acute and convalescent sera who had experienced a single infection and who received no specific therapy.

crease in antistreptolysin demonstrated in the convalescent sera. Thus, some of these 5 patients must have acquired a streptococcal infection at least one week prior to the observed illness. Since the numbers of patients in each time interval were small, the 107 patients with intervals of 35 days or less were divided into two groups, one with initial antistreptolysin titers of 200 units or greater and the other with titers of less than 200 units. The avereage latent period for the group with initial titers above 200 units was 16 days. The average period of the group with initial titer below 200 was 19 days, a differenece which is not significant.

The 38 patients who carried two different serologic types of group A streptococci did exhibit a different pattern of latent periods than the 127 patients who showed only one type in the two cultures. There were no

patients with a latent period of less than 8 days. However, for technical reasons it might have been difficult to establish the presence of two infections within a week of the observed respiratory illness. There was an increased number of patients with long latent periods; 21 cases or 55 per cent of the total group developed rheumatic fever more than 35 days after the onset of the observed illness. Of this group of 21 cases with a prolonged latent period, 7 had received an antibiotic as therapy for the observed streptococcal infection, whereas only 2 of the remaining 17 cases were given specific treatment. The second, streptococcal infection, usually inapparent clinically, was probably related directly to the rheumatic attack and accounted for the prolonged latent period in many of these patients.

The distribution of the latent periods of individuals who received antibiotics but showed no bacteriological evidence of an intervening new streptococcal infection is considerably different than the intervals exhibited by those 127 patients who received no therapy. Of the former, 26 per cent, as compared to 9 per cent of the latter group, exhibited latent periods of 8 days or less. The antistreptolysin titers of the acute phase sera from the 5 patients developing rheumatic fever at about the same time as the streptococcal respiratory illness began were 83, 83, 250, 317, and 625 indicating that in all probability 2 or 3 patients had recently experienced a streptococcal infection. Since some patients early in the course of acute rheumatic fever experience a recurrence of the symptom, soreness of the throat, it is possible that some of these short latent periods are an artifact. In addition, it should be recalled that approximately half of all patients with respiratory infections received antibiotics, so that if therapy did not alter the incidence of rheumatic fever in those patients who were destined to develop this complication after a very short latent period, the actual number observed in this category should approximate the number observed in the group of 127 patients who experienced a single infection and received no therapy. The figures show that there were 11 cases in both groups whose latent period was less than 8 days. Thus, it appears probable that treatment of the streptococcal infection is not likely to decrease the attack rate of rheumatic fever in patients with short latent periods.

The distribution of the latent periods in those patients whose initial culture failed to show group A streptococci is difficult to explain. Of the 6 patients developing rheumatic fever during the first time interval, streptococci were isolated from 4 a few days later. The initial antistreptolysin titer was available from 5 of the 6 patients. In 3 it was 83, and the re-

maining initial titers were 100 and 250. Of the total group 18 showed a positive culture during the latent period. Some of these acquisitions probably represent a new infection, accounting for the relatively large number of patients with long latent periods.

DISCUSSION

Our concept of the latent period in acute rheumatic fever has been derived from historical information obtained from the patient at the time the rheumatic symptoms appeared rather than from observations of patients with streptococcal infections who subsequently developed signs of rheumatic activity. Data obtained by the former method tend to be inaccurate since the memory of the patient concerning events occurring several weeks earlier is frequently poor, and the etiology of the preceding respiratory illness cannot be defined. In addition, the studies of Rantz, Boisvert and Spink[4] indicate that reinfection with a second type of group A streptococcus may occur during the latent period without associated overt clinical symptoms. Further errors are likely to occur because at least 14 per cent of patients with acute rheumatic fever give no history of a preceding illness and in another 24 per cent the streptococcal illness is so mild that the details are soon forgotten.[10]

In the population of 565 patients with acute rheumatic fever 48 per cent had been observed previously with an acute respiratory illness of sufficient severity to require hospitalization. In all but 23 an accurate history of the time of onsets of the streptococcal infection and rheumatic fever were obtained. Since routine cultures were obtained at the beginning of the respiratory illness, again three weeks later, and at the time of appearance of acute rheumatic fever, reinfections with new types of streptococci could be documented. Thus, it was possible to gain an accurate description of the latent period in a large group of patients. Such data provide information of both theoretical and practical value.

The normal distribution curve of the duration of the latent periods was based on 127 patients who received no therapy. The majority was thought to have experienced a single preceding streptococcal infection as determined by routine bacteriological studies. It was recognized that a few patients exhibiting a very short latent period may have actually acquired a clinically inapparent infection some weeks prior to the observed respiratory illness, and the observed illness actually represented the recurrence of sore throat which some patients with acute rheumatic fever experience. In favor of this interpretation is the high titer of antistreptolysin in the acute phase serum of some patients showing a short latent period. In

addition, it appeared reasonable to assume that the majority of patients who exhibited a latent period of over 35 days probably had experienced a second, clinically inapparent streptococcal infection which was not detected by the techniques employed.

The length of the latent period did not appear to be related to the type of streptococcus responsible for the respiratory illness nor did it appear to vary with the magnitude of the antibody response to infection as measured by antistreptolysin. There was a slight difference in the average latent periods of those patients with an initial antistreptolysin titer of less than 200 units and those with greater than 200 units. The average periods were 19 and 16 days, respectively. This difference may have been produced by a few patients who had experienced an inapparent streptococcal infection a few days or weeks prior to the observed illness. Thus, the initial antistreptolysin titer would be high and the latent period short in such patients. Since the difference in the latent periods between the two groups was not great, it may be concluded that few such cases were included in this analysis.

Of the 127 patients, 21 were experiencing a recurrent attack of acute rheumatic fever, and in these there was no indication that the latent period was shortenend. It has been reported that recurrent attacks are not associated with shorter latent periods[11] but no published data are available to support such a statement. More extensive information on this subject would be valuable since rheumatic fever has been compared to serum sickness in which the readministration of the responsible antigen results in an accelerated reaction.

As indicated earlier, Rantz, Boisvert and Spink[4] suggested that reinfection with a new serological type during the latent period might be causally related to the development of acute rheumatic fever. The present analysis does not permit a comparison of attack rates in patients with one and two infections. However, some information is available from a study of reinfections in 5198 patients with streptococcal infections observed at the same military installation between January 1949 and January 1954[8] All of these patients received antibiotics and only a *single* convalescent culture was obtained from each. The incidence of reinfection was 10 per cent, whereas in the present study 15 per cent of the patients with rheumatic fever showed two organisms during the latent period. Reinfections were especially prevalent among those patients with long latent periods. Indeed, if only those patients with latent periods of less than 36 days are considered, 9 per cent experienced a second infection, a figure which is similar to the 10 per cent figure observed in the population quoted above.

Thus, the present data do not support the view that two closely spaced infections play a major role in the causation of rheumatic fever.

The curve showing the distribution of cases of rheumatic fever according to the length of the latent period should be of practical value to the pediatrician, internist, and family physician. It is a well established fact that proper therapy of the streptococcal infection with penicillin will prevent acute rheumatic fever and, presumably rheumatic carditis. Treatment initiated at the onset of symptoms of the streptococcal infection might be expected to fail to eliminate those attacks of rheumatic fever that develop

TABLE 6. MAXIMAL POSSIBLE EFFECT OF THERAPY OF STREPTOCOCCAL INFECTIONS ON THE OCCURRENCE OF RHEUMATIC FEVER ACCORDING TO THE TIME OF INSTITUTION OF TREATMENT

Time therapy instituted (days)*	*Per cent reduction of acute rheumatic fever†*
0	98
8	90
14	67
21	42
29	8

* Expressed in days from onset of first symptom of the respiratory illness.
† Calculations are based on the assumption that adequate therapy will prevent all rheumatic fever except those cases occurring through the second day. Rheumatic fever developing after 35 days is considered not to be affected by therapy.

in the first two days. This represents approximately 2 per cent of all rheumatic attacks. The present data as well as those obtained during the course of another study[9] support this conclusion. More important, however, is the fact that therapy with penicillin will prevent rheumatic fever even when it is administered after the patient has fully recovered from the immediate effects of the streptococcal infection. In the only study reported,[32] penicillin therapy was initiated nine days after the onset of the acute respiratory illness at a time when the patients were asymptomatic. The reduction in the attack rate of rheumatic fever in those developing symptoms between 10 and 35 days was 82 per cent as compared to a control group of patients.

If the assumption is made that *adequate* therapy of the streptococcal infection instituted at any time during the latent period will prevent all subsequent attacks of rheumatic fever except those developing within the first 2 days and those appearing after an interval of 35 days, then therapy initiated at the times indicated in Table 6 will reduce the total number of

cases of rheumatic fever by the amounts indicated. These data would indicate that the physician who makes a diagnosis of a streptococcal infection in one patient should then determine the dates of onset of sore throat in other household contacts. Those contacts whose respiratory symptoms developed during the preceding month and who received no specific therapy during the acute illness should be placed on treatment with penicillin for the prevention of rheumatic fever. Ideally, oropharyngeal cultures from all contacts should also be obtained, since some infections will produce no symptoms and such individuals should receive the benefit of specific therapy.

SUMMARY

The latent period between the onset of the streptococcal infection and the onset of acute rheumatic fever was determined in 251 patients. In 113 of these patients an infection by a single type of streptococcus occurred and the patient received no specific therapy. In this group the mean latent period was 18.6 days. There was no correlation between the type of group A streptococcus causing the infection and the length of the latent period. Patients who had experienced a prior attack of rheumatic fever did not exhibit an accelerated onset of rheumatic symptoms. The length of the latent period did not appear to be related to the magnitude of the antistreptolysin response to infection.

Reinfection with a new type of streptococcus during the latent period did not prove to be important in the causation of rheumatic fever, but it was associated with prolongation of the latent period in many instances.

On the basis of this analysis it is recommended that patients with streptococcal infections should receive therapy with penicillin for the prevention of rheumatic fever even though the onset of respiratory symptoms was as long as four weeks previously.

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ALBERT B. SABIN*

The Children's Hospital Research Foundation, University of Cincinnati College of Medicine

POLIOMYELITIS IN BRAZIL, URUGUAY, ARGENTINA AND CHILE

Data of Importance in Planning for Elimination of the Disease

FOREWORD

It is a special pleasure for me to contribute something, even something so meager as the present communication, to the number of the Yale Journal of Biology and Medicine issued in honor of John R. Paul. It is 30 years since we first met and began, each in his own way, to study certain aspects of poliomyelitis. Together we have passed through an exciting era in the development of knowledge of this disease. The studies of John Paul and his associates greatly contributed to our understanding of the nature of poliomyelitis and to the ultimate goal of its elimination as a cause of human misery. The subject of the present communication has also been of interest to Dr. Paul, who drew attention to the increasing occurrence of epidemics of poliomyelitis in South America in his article concerned with endemic and epidemic trends of poliomyelitis in Central and South America.[1]

* * *

INTRODUCTION

Recent epidemiologic and laboratory studies have provided evidence that in orally administered, live, attenuated poliovirus vaccine we now have a tool with which the complete elimination of poliomyelitis can be undertaken.[2-7] The public health authorities of several countries in South America have recently decided to initiate nation-wide programs of immunization with oral poliomyelitis vaccine, and I was invited to consult with them about various aspects of such a program.

The basic information that one needs for deciding on the type of program that is most suitable for a given country includes data on i) incidence of the disease in recent years, with special reference to determining whether or not there is a pattern suggesting a regular periodicity for years of high and low incidence, ii) the age distribution of the reported cases in recent years as an index to the extent of natural immunization and to the age groups which should be included in the initial nation-wide

* Professor of Research Pediatrics.

program, and iii) the seasonal incidence of the reported cases as an index to the months of the year that may be optimum for community-wide programs. The purpose of this communication is to report this type of information for Brazil, Uruguay, Argentina and Chile and to indicate the kind of vaccination program that seemed most advisable for each of these countries.

BRAZIL

At the Fourth International Poliomyelitis Conference held at Geneva in 1957, Prof. José Martinho da Rocha[8] presented some data on poliomyelitis

TABLE 1. MONTHLY ADMISSIONS OF NEW CASES OF ACUTE POLIOMYELITIS TO ONE HOSPITAL (JESUS) IN RIO DE JANEIRO, 1952-1961*

Month	*1952*	*1953*	*1954*	*1955*	*1956*	*1957*	*1958*	*1959*	*1960*	*1961*
January	14	24	4	6	3	12	48	25	25	24
February	0	56	7	9	5	5	13	9	12	15
March	4	68	13	11	13	16	15	7	17	31
April	2	73	21	3	20	15	8	17	29	34
May	8	108	57	12	16	11	6	9	35	65
June	6	82	43	9	18	3	8	8	61	
July	1	49	44	9	34	7	15	9	38	
August	5	17	24	9	50	15	28	4	19	
September	5	16	13	9	40	10	37	11	18	
October	6	6	12	10	35	25	55	11	15	
November	18	4	15	13	36	32	87	6	10	
December	26	6	4	11	14	69	46	5	19	
Total	95	509	257	111	284	220	366	121	289	—

* Data supplied by Dr. Oswaldo P. Campos.

in Brazil, and called attention to the absence of exact statistical information. This absence of adequate statistics still obtains, but an idea of the relative incidence of the disease in different years, months, and age groups can be obtained from the hospitals to which patients are admitted during the acute phase of poliomyelitis. The data obtained from only one such hospital in Rio de Janeiro, which were made available to me by Dr. Oswaldo P. Campos are shown in Table 1. In the southern hemisphere, one obtains a better idea of the annual fluctuation in the number of cases by grouping the data for the periods of 1 July to 30 June than for the periods of 1 January to 31 December, and this was done in Table 2 with the data from the Jesus Hospital in Rio de Janeiro. It can be seen that while during

the past nine years there were two peak periods in 1952-1953 and in 1958-1959, the incidence of admissions during the intervening years is not strikingly lower than the peak years. It is noteworthy, that in the incomplete statistics of the Federal Health Service, the city of Rio de Janeiro is reported as having had 37 cases in 1952, 746 cases in 1953, and 357 cases in 1954. In the city of São Paulo, according to the same statistics, the number of cases reported annually from 1944 to 1952 ranged from 3 to 29, with 136 in 1953, 42 in 1954, and 127 in 1955. During my visit to São Paulo I was informed that about 1200 cases of paralytic poliomyelitis were admitted to the hospitals of the city in 1960, and that in the years immediately preceding 1960 the admissions ranged from 400 to 500. There was no indication in the data presented to me how many of these patients were from outside the city of São Paulo, but there was general agreement that poliomyelitis had become a much more important problem during the past five years.

TABLE 2. VARIATIONS IN ANNUAL INCIDENCE (JULY 1-JUNE 30) AND IN MONTHS OF HIGHEST INCIDENCE OF POLIOMYELITIS IN RIO DE JANEIRO—BASED ON ADMISSIONS TO JESUS HOSPITAL

Period of July 1-June 30	*Total no. admitted*	*Months with 20 or more cases*	*Peak month and no. of cases*
1952-1953	472	XII, I, II, III, IV, V, VI	V-108
1953-1954	243	VII, IV, V, VI	V- 57
1954-1955	162	VII, VIII	VII- 44
1955-1956	136	IV	IV- 20
1956-1957	271	VII, VIII, IX, X, XI	VIII- 50
1957-1958	256	X, XI, XII, I	XII- 69
1958-1959	343	VIII, IX, X, XI, XII, I	XI- 87
1959-1960	216	I, IV, V, VI	VI- 61
1960-1961 (11 months)	288	VII, I, III, IV, V	V- 65

Rio de Janiero at about 23° south latitude has a climate which is rather similar to that of Miami, Florida at about 26° north latitude. The data shown in Table 2 indicate that while the peak months of incidence may occur more often during the hot months of November to May than during the cooler months of June to October, there is no regularity in different years. Ayroza Galvão *et al.*[9] reporting on poliomyelitis in the interior of the state of São Paulo, which occupies about the same latitude as Rio de Janeiro, showed that for the period of 1942-1951, the hot months

of November to May had more poliomyelitis than the cooler months of June to October, but they also pointed to irregularities during outbreaks in 1952 and 1953.

At the Fourth International Poliomyelitis Conference, Dr. Oswaldo P. Campos[10] reported that among 1171 new cases of poliomyelitis of known age that came under his personal observation from June 1954 to May 1957 in Rio de Janeiro, 59% were under one year of age, 88% under three years, and 94% under five years of age. Somewhat similar data for Rio de Janeiro in 1954 were reported by Martins da Silva and Syverton[11] (see Table 3). Dr. Campos also pointed out to me that because of this high incidence of infection with polioviruses during the first year of life,

TABLE 3. AGE DISTRIBUTION OF REPORTED CASES OF PARALYTIC POLIOMYELITIS IN BRAZIL

Source of data	*Region*	*Period*	*No. of cases*	*Per cent under 3 yrs.*	*Per cent under 5 yrs.*
Martins da Silva and Syverton	Rio de Janeiro	1954	356	83.6	91.8
Campos	" " "	June '54-May '57	1171	88.1	93.8
Galvão	São Paulo—urban	1949-1953	533	83.	90.
	" " —rural	" "	663	71.	88.

the milder cases of paralysis are not noticed until the child begins to walk or differences in growth of the affected and normal extremities become apparent. Accordingly, the relative proportion of children acquiring the disease during the first year of life is probably much higher. Borges[12] in a recent report quoted a personal communication from Doctors A. M. Santos and W. V. Silva of the Orthopedic Department of the Hospital of the Faculty of Medicine of the University of São Paulo, that about 56% of their poliomyelitis patients were under one year of age and 90% under two years of age. Somewhat lower percentages are reported for the urban and rural areas of the interior of the state of São Paulo by Ayroza Galvão *et al.*[9]

Payne[13] has previously considered the agreement that may be expected and has been observed between estimates of susceptibility of certain populations based on age incidence of reported cases and on serological surveys. He indicated that while in most reported instances direct comparisons were not possible, there was a suggestion that the incidence of sus-

ceptibles at a given age based on demonstrable antibodies for each of the three types was higher than that based on age distribution of reported cases. Payne called attention to the possibility that infection with one type can exert partial protection against the paralytic effects of another type; this has been demonstrated both experimentally and epidemiologically, and may account for the fact that the age below which 90% of all paralytic cases have occurred, may on serologic survey still show 20 to 30% of susceptibles to type 1 poliovirus or to one of the other 2 types. Table 4 presents data calculated from the results obtained by Martins

TABLE 4. INCIDENCE OF ANTIBODY FOR EACH OF 3 TYPES OF POLIOVIRUS IN CHILDREN OVER 6 YEARS OF AGE IN DIFFERENT SOCIOECONOMIC GROUPS IN RIO DE JANEIRO —JULY TO AUGUST, 1954*

Socioeconomic group	*Average age years*	*No. tested*	*Per cent positive for type*				
			I	*II*	*III*	*I + II + III*	*One or more types*
A-low	8.6	29	96.6	96.6	82.8	79.4	100
B-average	11.8	58	77.6	77.6	70.7	53.4	96.6
C-high	18.3	24	66.7	75.0	41.7	29.1	95.8

A—⅔ of children from city slums and ⅓ from semi-rural school; overcrowding, no running water or sewage disposal.
B—Children from small houses or apartments with running water, sewage disposal and no overcrowding.
C—Children from upper income brackets—both Brazilian and foreign.
*Tabulated from data in Martins da Silva and Syverton.[11]

da Silva and Syverton[11] in a serology survey performed on children of different socio-economic groups in Rio de Janeiro in 1954, i.e. after 2 years of relatively high incidence of the disease. It is noteworthy, therefore, that among 58 children over 6 years of age (average age of 11.8 years) from the "average" socio-economic group, there were still about 22% without antibody for type 1 or type 2, and 29% without antibody for type 3. Yet about 84% of the reported cases were under 3 years of age and 92% under 5 years of age during the period immediately preceding the serologic survey. Furthermore, Martins da Silva and Syverton[11] reported that 60 to 70% of all the cases came from the higher than average socio-economic groups, and Campos[10] also stressed the fact that paralytic poliomyelitis was rare among the children living in the slums of Rio de Janeiro. It would appear, therefore, that as in the data analyzed by Payne,[13] the possession of antibody against at least one of the 3 types of poliovirus

(observed in 97% of Rio children in group B of Table 4) constitutes a better index to susceptibility of a given population to paralysis than the incidence of antibody for type 1, the most common paralytogenic type.

Without having any statistics on the proportion of the population in Rio de Janeiro and São Paulo that lives in slums, I gathered the impression, while driving about these cities, that the majority of the population lives in very fine apartments and small homes, and quite a large portion of the population in luxurious apartments and homes — and yet it would appear that even under these conditions, the natural dissemination of polioviruses is so extensive that at least 90% of all the reported cases of paralytic poliomyelitis continues to occur in children under 5 years of age. The amount of Salk vaccine used in Brazil since 1956 has been comparatively small[10] and it is doubtful that it has had a significant effect on the age incidence of the disease. Since extensive dissemination of polioviruses under such conditions is undoubtedly also associated with extensive dissemination of other enteric viruses, as shown in the study in Toluca, Mexico, it seemed advisable to recommend a vaccination program for Brazil that was based on what was learned in Toluca.[2] This involves the administration of two doses of trivalent vaccine, with a minimum of $10^{5.3}$ TCD_{50} (50% tissue culture infective doses) or 10^5 PFU (plaque forming units) of each of the 3 types per dose, to all children from 6 weeks to under 5 years of age, at an interval of about 6 to 8 weeks. Since, under the subtropical and tropical climatic conditions obtaining in most of Brazil, there is no season that is free of extensive dissemination of polioviruses and other enteric viruses, the oral vaccine program can be started at any time of the year. It is conceivable that in the southernmost states of Santa Catarina and Rio Grande do Sul, where truly cold weather may occur during the months of June to October, there may be an advantage to performing the mass vaccination programs during these months. Every attempt is to be made to reach at least 70 to 80%, and if at all possible more, of the children in the designated age group, and to complete the vaccine feeding within about a week or less in a given town, village, or subdivision of a large city. After the conclusion of the community-wide program in a given area, arrangements are to be made for immunizing of the new generations of children during the first 6 months of life by giving the first dose of trivalent vaccine at 2 to 3 months of age (regardless of the time of the year that a child may reach that age) and the second dose of trivalent vaccine at 4 to 5 months of age. The administration of the vaccine in trivalent, instead of monovalent form, to these children is based chiefly on administrative considerations. Private physic-

ians, seeing children regularly during their first year of life, may be urged to give the individual types separately at 6-week intervals (in the order of I—III—II) with a booster dose of trivalent vaccine at 10 to 12 months of age. It may be advisable for private physicians to administer the trivalent vaccine to persons in the higher socio-economic groups up to 20 years or more, since about 71% of the older children of this group were found to be without antibody for one or another of the 3 types of poliovirus (see Table 4).

TABLE 5. ANNUAL INCIDENCE OF PARALYTIC POLIOMYELITIS IN URUGUAY BASED ON CASES REPORTED FROM JULY 1-JUNE 30

Period of July 1-June 30	*No. of cases*
1950-1951*	131
1951-1952	18**
1952-1953	57**
1953-1954	42**
1954-1955*	550
1955-1956	92
1956-1957	65
1957-1958	44
1958-1959	174
1959-1960	13
1960-1961	36

*Based on data reported by Leunda, *et al.*[15]

** These data are for period of June 1-May 31, from document by Dr. Oswaldo Luzardo of the Ministry of Public Health.

Remaining data are based on document of Ministry of Public Health giving monthly incidence of reported cases of paralytic poliomyelitis.

URUGUAY

At the Third International Conference on Poliomyelitis it was reported that only about 15 to 20 cases had occurred in Uruguay during the last few years.[14] Actually at least 248 paralytic cases are on record for the period of July 1, 1950 to June 30, 1954 in the official statistics of the Ministry of Health, and during the very next 12 months, between July 1, 1954 and June 30, 1955, a total of 550 paralytic cases was reported for this country with a population of about 2.6 million—a real epidemic with an attack rate of about 21 per 100,000. A more accurate history of poliomyelitis in Uruguay from 1906 to 1959, is contained in the report presented by Dr. Juan J. Leunda[15] for the National Committee against Poliomyelitis of the Ministry of Public Health at the First International Con-

ference on Live Poliovirus Vaccines (Washington, D.C., 1959). In order to obtain an idea of a possible periodicity in years of high and low incidence of the disease, the available data for 1950-1961 were arranged on the basis of incidence during the 12-month periods between July 1 and June 30 (see Table 5). It may be seen that periods of higher incidence recurred every 4 years since July 1, 1950. The unusually low figure of 13 cases for the period of 1959-1960 is undoubtedly related to the extensive use of live poliovirus vaccine (Lederle strains), principally in Montevideo, during the nation-wide epidemic of 1958-1959. It is noteworthy that of the 13 cases reported for the entire country between July

TABLE 6. AGE DISTRIBUTION OF PARALYTIC POLIOMYELITIS IN URUGUAY, 1955-1960

Period	*Total no.*	*Per cent in indicated group*				
		0-4	*0-9*	*0-14*	*0-19*	*20+*
1955	551	60.	77.	87.3	92.4	7.5
1956 + 1957	124	60.	77.	85.5	90.3	9.7
1958	166	60.	72.	81.0	88.0	12.0
1959 + 1960	73	38.	54.	68.5	80.0	20.0

Prepared from data made available by Dr. Alberto Bertolini, Director de Division Higiene, Ministerio de Salud Publica, Uruguay.

1, 1959 and June 30, 1960, only 1 was reported from Montevideo (population of about 1 million). At the same time the data for 1960-1961 indicated that the number of cases was again rising, and that in the absence of a vaccination program another epidemic could be expected within 2 years.

The age distribution of the reported cases since 1955, shown in Table 6, indicates a very much different picture than in Brazil. The age below which about 90% of the cases occurred in Uruguay was in the range of 20 years until 1958. In 1959 and 1960, only 80% of the cases were under 20 years, and 92% under 30 years. The possibility was considered that the extensive vaccination program in Montevideo in 1958-1959 may have protected relatively more persons under 20 years of age, but the data on the age incidence of the cases in Montevideo and in the remainder of Uruguay were not immediately available during my visit.

Uruguay is located between 30° and 35° south latitude and the months of June to October are characterized by distinctly cold and cool weather. The data on the monthly incidence of cases, shown in Table 7,

indicate that particularly during the years of higher incidence, the peak months were the hot months of December to April, except for 1958 which presented a somewhat different pattern.

Previous Use of Poliomyelitis Vaccine in Uruguay — According to the official data of the Ministry of Public Health relatively little Salk vaccine has been used. The number receiving 3 or more doses was as follows: 1956 — 0; 1957 — 26,424; 1958 — 18,805; 1959 — 14,657; 1960 — 8,449.

TABLE 7. MONTHLY INCIDENCE OF PARALYTIC POLIOMYELITIS IN URUGUAY*

Period of	*Month*												*Total no.*
July 1-June 30	*VII*	*VIII*	*IX*	*X*	*XI*	*XII*	*I*	*II*	*III*	*IV*	*V*	*VI*	*of cases*
1950-1951**	1	1	4	4	6	22	44	26	11	5	3	4	131
1954-1955**	2	4	5	5	3	28	56	90	211	104	35	7	550
1956-1957	3	6	5	2	3	8	5	8	4	7	10	4	65
1957-1958	3	2	2	0	2	2	1	4	7	4	9	8	44
1958-1959	8	7	5	17	43	49	23	10	7	4	1	0	174
1959-1960	1	3	1	0	2	3	0	0	2	1	0	0	13
1960-1961	1	1	1	2	0	2	4	6	8	4	5	2	36

* Prepared from document of Ministry of Public Health giving monthly incidence of reported cases of paralytic poliomyelitis.
**Data in report by Leunda, *et al.*[15]

Some of the data on the mass vaccination program with live attenuated poliovirus vaccine (Lederle) in Montevideo have already been reported,[15] but there was considerable discussion among certain groups in Uruguay about the effect of this program on the outbreak of 1958-1959 and on the planning of a new, nation-wide program of vaccination with oral vaccine. The vaccination program was at no time truly coordinated. From May to September, 1958, when the incidence of cases was higher than usual for these cold months of the year (see Table 7), only about 18,000 persons received the vaccine usually in the order of II—III—I from private physicians on an individual basis. In October, 1958, when a further increase in cases signalled the premature beginning of an epidemic, which might have been expected on the basis of the periodicity shown in Table 5, vaccination was begun on a much larger scale but still without coordination, in the sense that it was spread over many months during which period some were getting type 2, while others were getting type 3, and still others type 1. The epidemic, later shown to be caused predominantly by type 1 virus, terminated in the midst of the hot season, but this was also

true of the 1950-1951 outbreak even though it began in December (see Table 7). It is noteworthy that the 1958-1959 outbreak terminated during the hot season also in the remainder of Uruguay where little or no vaccine was employed. According to the published data[15] it would appear that only about 50% or less of the young children in Montevideo received the vaccine, which, taken together with its administration in an uncoordinated manner over a period of many months, cannot be regarded as a proper way to use oral vaccine in the face of an epidemic. There can be little doubt that many cases of paralytic poliomyelitis were prevented by the vaccination. According to Dr. Juan C. Bacigalupi of the University Institute of Hygiene in Montevideo, there were no cases among the approximately 300,000 persons who received the type 1 oral vaccine. The following considerations influenced the decision to include the persons who received the oral vaccine in 1958-1959 in the nation-wide program that was being planned for 1961:

a) most of the persons received the vaccine during the warm and hot months of the year, during a type 1 outbreak, and in an uncoordinated protracted manner — all of which may have contributed to interference with the optimum multiplication of the vaccine strains in an unknown proportion of the people;
b) the type 2 Lederle strain is not highly immunogenic even in the absence of interference;
c) refeeding these people will help increase the number of resistant intestinal tracts in the community, and should thus greatly improve the chances of breaking the chain of transmission of the naturally occurring polioviruses;
d) revaccination would eliminate administrative complications.

Plan for Nation-wide Oral Vaccine Program in 1961. — The age incidence and the seasonal incidence of poliomyelitis in Uruguay, suggested that the conditions were comparable to those obtaining in North American cities like Cincinnati,[8] where the 3 types of oral vaccine were administered separately to the pre-school and school children up to 18 years of age, in the order of I—III—II. For Uruguay, the period of June to November was optimum, and the age groups to be included in the first phase of the program under the direction of the Ministry of Public Health would be from 6 weeks to under 20 years. It was determined that the program should be coordinated in such a way that the same type of vaccine would be administered to all during a period of 7 to 10 days in any one region and that this would be followed by a period of about 4 weeks when no vaccine would be administered, before the next type is to be given in a similar

manner. Phase two of the program, dealing with the immunization of the oncoming generations of children, takes into consideration the extensive availability of medical service to children during their first year of life through private physicians and clinics. For example, Montevideo (population about 1 million) alone has about 200 pediatricians, which is three times as many as there are in Cincinnati and adjacent communities with a comparable population. The procedure recommended here was to integrate the administration of oral poliovirus vaccine into the existing schedule of immunizations against diphtheria, pertussis, tetanus and smallpox, and to administer each type separately in the order of I—III—II at the same time as the other immunizations are given, without reference to the season of the year. Since this would involve administration of one or another type of the vaccine during the hot months of the year, when interference from other intestinal viruses is more likely to occur in some instances, it was also recommended that a trivalent dose of vaccine be given between 10 and 12 months of age, at a time when the child is brought for a booster injection of diphtheria-pertussis-tetanus vaccines.

ARGENTINA

At the Third International Poliomyelitis Conference (Rome, 1954), Dr. Bismarck Lucero[18] reported on the incidence of poliomyelitis in Argentina up to 1953, and called attention to the fact that the *majority* of cases occurred in children between 1 and 7 years of age. I am indebted to Dr. Lucero and to Dr. Ignacio Pirosky, director of the Institute Nacional de Microbiologia, Ministerio de Asistencia Social y Salud Publica, for making available to me the data presented in Tables 8, 9 and 10. In 1956, Argentina had its largest epidemic with a total of 6,496 paralytic cases in a population of about 19.5 million, an attack rate of 33.3 per 100,000. If a comparable epidemic had occurred in the U.S.A. with a population of 180 million, there would have been 60,000 paralytic cases. The highest recorded incidence in the U.S.A. of 37.2 per 100,000 was in 1952 but this also included so-called nonparalytic cases, and it is highly probable that the attack rate for paralytic cases was closer to 20 per 100,000. The monthly incidence data for the past 10 years were not immediately available, and I therefore could not analyze the incidence of the disease on a July 1—June 30 basis which is more illuminating in the search for periodicity in the southern hemisphere. The January—December data for 1949-1956, shown in Table 8, suggest a 2 to 3-year periodicity for varying degrees of increase in incidence. I did not obtain precise data on how much Salk vaccine has been used in Argentina in recent years nor on

what proportion of the population had received 3 or more doses. However, Salk vaccine is bought by the government from the lowest bidders, and these bids are a matter of public record. Late in 1959 the government obtained bids on 2 million doses (c.c.), in 1960 on 3.8 million doses, and in April 1961 on 5 million doses.* In 1959 the total number of reported paralytic cases was 1,050 and in 1960 it wa sagain about 1,000.

TABLE 8. PARALYTIC POLIOMYELITIS IN ARGENTINA, 1942-1959

Year	*No. of cases*	*Cases/100,000*
1942	1338	9.1
1943	1556	10.6
1944	497	3.3
1945	357	2.5
1946	659	4.2
1947	467	2.9
1948	552	3.6
1949	1103	6.5
1950	586	3.4
1951	1015	5.7
1952	695	3.8
1953	2579	14.0
1954	871	4.6
1955	435	2.3
1956	6496	33.3
1957	760	3.8
1958	842	4.2
1959	1050	5.1

Data from "Epidemiologia de la poliomielitis—formas paraliticas—ano 1958", Ministerio de Asistencia Social y Salud Publica (Argentina), Seccion Estadisticas Vitales, Buenos Aires, 1959.
Data for 1959 from official statistics of Health Ministry.

The age distribution of reported cases for 1954-1959, shown in Table 9, indicates that the 90% incidence has fluctuated from under 13 to under 10. It is noteworthy, however, that in 1958 and 1959, about 81 to 83% of all cases were under 5 years of age.

Argentina includes territory lying between 22° and 55° south latitude, with the majority of the population residing in the middle zone. Data for

* It is noteworthy that in 1960, the contracts went to two American companies first at 10 American cents per dose and later at 7 cents per dose, and that in 1961 a European company obtained the contract by offering vaccine at 5 cents a dose—prices that are extraordinarily low by comparison with those obtaining in the U.S.A., Europe and other South American countries.

the monthly incidence of poliomyelitis were immediately available only for 1958, and these show (Table 10) that December to May are generally the months of highest incidence in the Buenos Aires area at 34.5° as well as in Tucuman at 26° south latitude.

In considering various aspects of a nation-wide oral poliovirus vaccine program for Argentina, there was no difficulty in deciding a) that the optimum time would be the cool and cold months of the year, i.e. June to November, and b) that children up to at least 9 and preferably 12

TABLE 9. AGE DISTRIBUTION OF REPORTED CASES OF PARALYTIC POLIOMYELITIS IN ARGENTINA, 1954-1959

	Per cent of total no. of known age in indicated year					
Age group	*1954*	*1955*	*1956*	*1957*	*1958*	*1959*
Under 2 yrs.	41.8	41.4	43.6	32.7	56.4	53.6
" 5 "	71.0	74.4	74.6	72.7	80.7	83.1
" 10 "	86.0	90.0	87.2	86.6	90.2	91.8
" 13 "	90.5	94.3	90.5	89.9	93.9	93.7
" 15 "	93.8	95.3	92.2	91.7	94.8	94.2
" 20 "	95.3	97.3	94.8	94.0	96.6	96.2
" 25 "	97.4	98.6	96.8	96.5	97.9	97.1
Total no. of known age	810	311	6496	760	842	1050

Prepared from data made available by Direccion de Enfermedades Transimissibles, Ministerio de Asistencia Social y Salud Publica, Republica Argentina.

years should be included in the primary community-wide phase of vaccination. What was more difficult to decide was whether the children of Argentina, with 80% of the cases under 5 years of age, represented a population with massive viral enteric infection throughout the year, winter and summer, for whom the Toluca model of two trivalent doses might be optimum, or whether the dissemination of intestinal viruses may be sufficiently suppressed during the winter season to expect that the separate administration of the 3 types of vaccine would yield the best results. It seemed to me that the distinctly high, summer incidence of poliomyelitis indicated that there was much less dissemination of polioviruses, and therefore also of enteric viruses generally, during the cold and cool months of the year— and that the situation in Argentina was certainly not significantly different from that obtaining in the low socio-economic groups in Cincinnati or among the children of Czechoslovakia or Hungary. Accord-

ingly, I recommended that the 3 types of oral vaccine be administered separately in the order of I—III—II, with intervals of at least 6 weeks between each type to allow for the maximum possible intrafamilial and interfamilial spread of the viruses, as a means of reaching those children who might have been in a refractory state because of infection with another enteric virus at the time they received the vaccine. The maintenance program in newborn children could follow the pattern recommended for the U.S.A. and Uruguay where the facilities for medical care of children

TABLE 10. MONTHLY INCIDENCE OF PARALYTIC POLIOMYELITIS IN ARGENTINA IN 1958

Region	*Total cases*	*Cases per 100,000*	*VII*	*VIII*	*IX*	*X*	*XI*	*XII*	*I*	*II*	*III*	*IV*	*V*	*VI*
Entire country	842	4.2	42	48	33	30	59	108	91	108	100	72	87	64
Buenos Aires	67	1.2	2	2	3	3	3	8	8	10	7	8	6	7
Districts adjacent to Buenos Aires	216	10.0	13	11	12	9	25	34	15	15	24	23	20	15
Tucuman	60	7.5	2	2	0	1	1	7	18	15	8	4	0	2

Data from document of Ministry of Health cited in Table 8.

during the first year of life permitted it, or could consist of the administration of several doses of trivalent vaccine where that would be administratively more feasible.

CHILE

The epidemiology of poliomyelitis in Chile up until 1954 was described by Horwitz, *et al.*,[17] and Borgoño[18] reported some of the characteristics of the epidemics in 1955-1956. I am indebted to Doctors J. M. Borgoño and C. Ristori of the Department of Epidemiology of the Servicio Nacional de Salud for the data presented in Tables 11, 12 and 13. Borgoño[18] stressed the fact that the official statistics on the incidence of poliomyelitis in Chile are incomplete because private physicians only rarely reported cases that are not admitted to the hospitals. An analysis of the incidence of poliomyelitis in Chile, for the 12-month periods between July 1 and June 30 for 1947-1961 (Table 11), suggests a trend toward a 4-year periodicity for years of higher incidence, excepting the period of 1953-1956 during which two high years occurred. The great increase in poliomyelitis in

recent years is most strikingly documented by the fact that during the 7 years from 1940-1946 only 343 cases were reported, with an avereage case fatality rate of 14%, while during the 14 years between July 1, 1947 and June 30, 1961 a total of 6,070 cases were reported with an average case fatality rate of 17.7%—and this for a country whose total population in 1955 was about 6.4 million and in 1960 about 7.6 million. The case

TABLE 11. REPORTED CASES OF PARALYTIC POLIOMYELITIS IN CHILE BASED ON JULY 1 TO JUNE 30 GROUPING

Period of July 1-June 30	*No. of cases*
1947-1948	147
1948-1949	121
1949-1950	800
1950-1951	216
1951-1952	480
1952-1953	443
1953-1954	773
1954-1955	316
1955-1956	815*
1956-1957	336
1957-1958	356
1958-1959	367
1959-1960	529
1960-1961	371

Data supplied by Servicio Nacional de Salud.

* In an article published by Dr. J. M. Borgoño, of the Department of Epidemiology, Servicio Nacional de Salud, in Revista del Servicio Nacional de Salud, 1: 207-221 (Dec.) 1956, the total number of cases for the period of October, 1955, to May, 1956, is given as 864. Dr. Borgoño explained that his published data were obtained by him directly from the records of the different hospitals, and that official reports by the hospitals to the National Health Service are not always complete.

fatality rates for 1958, 1959 and 1960 were 23.8%, 21.9% and 18.5% respectively. These continuing high case fatality rates since 1940, suggest, on the one hand, that this increase in poliomyelitis is not due to better reporting, and, on the other, that perhaps only 50% of the total number of paralytic cases are being reported—unless poliomyelitis in Chile is for some reason more often fatal than in other countries. Dr. Hernan Romero,[19] the official delegate of Chile to the Third International Conference on Poliomyelitis (Rome, 1954) said: "The reporting of poliomyelitis in the general population is defective." Although the government has been purchasing Salk vaccine for sale to the population, I gathered the impression that a

relatively small proportion of the children have received 3 doses, and the incidence of poliomyelitis in the last few years does not show a significant deviation from former years.

The age distribution of the reported cases in 1956-1960, shown in Table 12, indicates that about 83% of the reported cases occur under 5 years of age, and about 93% under 10 years of age. There is also no evidence of a recent shift in age distribution of the cases. Although Chile

TABLE 12. AGE DISTRIBUTION OF REPORTED CASES OF PARALYTIC POLIOMYELITIS IN CHILE, 1956-1960

		Per cent in indicated age group (years)		
Year	*Total no. of cases*	*Under 1*	*Under 5*	*Under 10*
1956	719	24.3	86.5	94.9
1957	333	20.4	79.3	92.5
1958	328	21.3	83.8	93.0
1959	456	25.4	84.2	94.1
1960	546	23.1*	82.7*	92.1*
1959				
Zona Norte	31	19.3	87.1	96.8
Zona Central	282	26.2	83.0	92.2
Zona Sur.	143	25.2	82.5	93.0

Prepared from data supplied by Servicio Nacional de Salud.
* Percentages calculated on basis of 532 cases of known age.

extends from a latitude of 18° to 55° south, 90% of the population lives in the central and southern portions between 32° and 55° south latitude. An analysis of the age distribution of cases in the 3 zones of Chile in 1959 (Table 12) showed no significant differences. The monthly incidence of cases in Chile in recent years, shown in Table 13, indicates that the highest incidence is during the hot months of December to April. It is noteworthy, however, that 13 of the 20 cases in 1960 in Coquimbo province, which lies between 29° and 32°, were reported during the months of August to November.

The National Health Service had decided on a nation-wide oral poliovirus vaccine program and two of the main questions concerned the age groups to be included and whether to use two doses of trivalent vaccine or the 3 types of vaccine separately. The immediately available data, indicating that about 93% of the reported cases occurred under 10 years of age, suggested that including all children up to 9 years of age would

certainly be adequate, and that it would be reasonable to give the vaccine only to the age group in which 90% of the reported cases occurred. With regard to the dosage schedule, the same considerations arose here as were discussed for Argentina, and the decision was against giving the two trivalent doses. Administratively, however, two vaccine sessions were preferred to three, and it was, therefore, decided to give the Type 1 vaccine first and then a combination of the Type 2 and 3 vaccines for the second dose. This procedure yielded good results in the nation-wide program in Czechoslovakia[7] in 1960, and, according to Paul, Horstmann and their associates (personal communication), also a very good antibody response

TABLE 13. MONTHLY INCIDENCE OF PARALYTIC POLIOMYELITIS IN CHILE

Period of July 1-June 30	*No. of cases in indicated month*											
	VII	*VIII*	*IX*	*X*	*XI*	*XII*	*I*	*II*	*III*	*IV*	*V*	*VI*
1955-1956	20	13	18	31	53	100	174	135	121	70	50	30
1956-1957	22	21	11	16	17	45	51	35	40	30	21	18
1959-1960	30	16	9	35	28	75	90	55	71	62	30	24

Data supplied by Dr. J. M. Borgoño, Department of Epidemiology, Servicio Nacional de Salud.

among children in large families in New Haven, Conn., during the winter of 1960. The main concern in such a program is that the intestinal resistance and persistence of the antibody may not be as good as when the type 2 and type 3 vaccines are given separately. Accordingly it was decided that several hundred one and two year old children be tested for antibody before and at various times after this vaccination schedule and the results would help to determine whether still another dose of monovalent, bivalent or trivalent vaccine should be given later on. It should be pointed out that there was also an economic consideration since two doses of trivalent vaccine would cost twice as much as the two doses consisting of types 1 only and a mixture of types 2 and 3.

There was no question that the optimum time for community-wide programs in Chile was during the cool and cold months of June to November. It was also suggested that, if time permitted, the interval between administration of the Type 1 vaccine and the mixture of Types 2 and 3 should be at least 8 weeks, in order to provide the longest possible time for intrafamilial and interfamilial spread of the virus.

In discussing the type of program that would be most feasible for immunization of the oncoming generations of children during the first year of life, it became necessary to take into consideration the fact that in Chile breast-feeding usually stops at 3 months, and that after that time more than 50 per cent of the children obtain monthly rations of dried milk. It appeared desirable to take advantage of this circumstance and have the mothers bring the children for their poliovirus vaccine when they come to get the milk. Under these circumstances, it would be administratively impractical to keep track of which type of vaccine a given child had had, and much simpler to administer two or more doses of trivalent vaccine at monthly intervals, and a "booster" dose at about 10 to 12 months of age.

Questions of Interest Regarding Oral Poliovirus Vaccine Programs

During the course of discussions on oral vaccination programs, the following questions most often came under consideration:

Q. Why not give the vaccine to newborn children right after birth or before 6 weeks of age?

A. Recent studies carried out by many investigators indicated that at least 100 times the usual dose is needed shortly after birth to obtain a response in at least 85% of children, and even with this large dose the response may be still lower in breast-fed children. During community-wide programs there is no harm in giving the usual dose to children under 6 weeks of age, provided provisions are made for re-vaccination at a later age.

Q. Why must the vaccine be given initially in coordinated, community-wide programs during a short period of time rather than on an individual basis over a long period as in the case of the Salk vaccine?

A. Oral vaccine, given on the same basis as Salk vaccine, can immunize the individual but would be ineffective in rapidly eliminating the naturally occurring polioviruses from the community, and poliomyelitis would continue to occur in the remaining susceptible persons. Administration of the oral vaccine on a community-wide basis to at least 70% or more of the most susceptible young children, in a coordinated manner in the shortest possible time, has the following odjectives: 1) to make the largest number of intestinal tracts resistant to infection in the shortest possible time as a means of quickly breaking the chain of transmission of the naturally occurring polioviruses and thereby protect also those in the family or community who are not immune;

2) in temperate climates, where the different types of vaccine are given separately, to avoid the interference which may occur when more than one type of virus is spreading in the community; and 3) in subtropical and tropical climates where there is massive enteric infection with other viruses, the rapid administration of the trivalent vaccine to at least 80% or more of the children under 5 years, is essential for temporarily suppressing dissemination of the other enteric viruses and for allowing nature to help disseminate the vaccine strains of polioviruses.

Q. Why should oral vaccine be given to persons who have already had 3 or more doses of Salk vaccine?

A. Becausce 1) it will immunize those who have failed to acquire immunity or have lost it; 2) it will give a powerful boost to the immunity of those who have not had a natural immunizing infection; and 3) it is necessary to provide the resistance to infection in the intestinal tract which is required for elimination of the naturally occurring polioviruses from the community. This applies particularly to young children, who are the most important spreaders of polioviruses.

Q. When the 3 types of oral vaccine are given separately, why are they given in the order of I—III—II instead of I—II—III or some other order?

A. Type I is given first because this quickly provides immunity against the type of poliovirus that is responsible for at least 85% of all paralytic poliomyelitis and for most epidemics. Type III is given next not only because it is next in importance as a cause of paralysis, but also because it is easier to terminate a persisting Type III intestinal infection by feeding Type II vaccine, which is the most dominant strain among the 3 types of vaccine, than vice versa. Moreover, when the Type II is fed it helps to eliminate the persisting excretion of the Type I or Type III viruses in those who are also susceptible to Type II.

Q. What is the simplest way of handling and administering the vaccine in a community-wide program?

A. To avoid excessive shipping and storage costs it is best to obtain the vaccine in undiluted bulk, then dilute it so that the dose (at least $10^{5.3}$ TCD_{50}) is contained in 2 drops (0.1 ml.), and store the diluted vaccine in medicine dropper bottles at about -20°C. Under these conditions, the potency of the diluted vaccine is known to be maintained without loss for a period of at least 8 months. During the actual vaccination program the diluted vaccine can be kept in the clinic or

doctor's office in an ordinary refrigerator at about +5°C for at least 7 days. For children of 2 years or older, who can chew a piece of sugar, the simplest procedure is to put the 2 drops of diluted vaccine on a lump of sugar. For younger children, the 2 drops of vaccine are added to a teaspoonful of dilute simple syrup. Vaccine in the form of candy, as prepared and used by Professor M. P. Chumakov and his associates of Moscow, is very simple to use in older children, but it is more expensive to prepare.

Q. Is a long period of public information and education necessary to obtain the maximum public response for a community-wide program?

A. No. Experience with such programs in the U.S.A. and elsewhere has shown that it is best to be prepared with vaccine and complete plan of operations before beginning the intensive campaign of public information. The best results are obtained when the public is told when and where they will be able to get the vaccine in their own community or neighborhood, and when they are faced with the necessity of making a decision in a matter of days rather than weeks or months. One to two weeks of public information have sufficed in most areas.

SUMMARY

Poliomyelitis has become of increasing importance in recent years in Brazil, Uruguay, Argentina and Chile. The amount of Salk vaccine that has been used in these countries has not significantly reduced the incidence. In Brazil, 94% of all reported cases occur in children under 5 years of age, the disease occurs with only slight fluctuations in all months of the year, and years with twice or more the annual incidence are uncommon. The oral poliovirus vaccine program recommended for Brazil was, therefore, based on the Toluca model, in which 2 doses of trivalent vaccine are to be given with an interval of about 6 to 8 weeks to all children from 6 weeks to under 5 years of age, without reference to season. For the new generations of children, subsequent to the initial community-wide program, two doses of trivalent vaccine were recommended during the first 6 months of life—the first at 2-3 months and the second at 4-5 months.

In Uruguay, Argentina, and Chile there is a distinctly higher incidence of the disease during the hot months of December to April, and epidemic years have occurred with varying periodicity. The age below which about 90% of the reported cases occurred in recent years is in the range of 20 to 30 years in Uruguay, 10 to 13 years in Argentina, and probably 7 to 9 years in Chile. In these 3 countries, the optimum time for the initial

community-wide programs is during the cool and cold months of June to November. The age groups to be covered includes persons up to 20 years in Uruguay, up to 12 years in Argentina, and up to 7 or 9 years in Chile. In Uruguay and Argentina it seemed advisable to recommend the separate administration of each of the 3 types of vaccine, while in Chile it was decided, chiefly for administrative expediency, to give Type I first and a mixture of 2 and 3 about 8 weeks later. The type of maintenance program for the new generations of children was also different in Chile than in Uruguay and Argentina. General questions of interest in relation to the planning and organization of oral poliovirus vaccine programs are discussed.

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HERBERT A. WENNER*
MARGARET F. LENAHAN**

Department of Pediatrics, University of Kansas School of Medicine, Kansas City

PROPAGATION OF GROUP A COXSACKIE VIRUSES IN TISSUE CULTURES

II. Some Interactions Between Virus and Mammalian Cells†

In contrast with the 6 Group B Coxsackie viruses which readily induce a cytopathic effect (CPE) in tissue cultures, many of the Group A viruses have either failed to alter visibly cells propagated in cultural systems, or have required "adaptation" for such an effect. In the course of studies made during the last two years we have obtained CPE on primate cells cultured *in vitro* for all except 7 serotypes among the 24 Group A viruses.

HISTORICAL RESUMÉ

In 1950, Slater and Syverton[1] noted that Group A, type 4 (Minnesota strain) virus propagated through 24 successive passages in cultures of minced embryonic mouse cells. Shortly thereafter Robbins *et al.*[2] and Riordan *et al.*[3] while engaged in studies of poliomyelitis commented on the capacities of unidentified Group A viruses (type 9, and others) to produce CPE on cultured primate cells. One of these strains (Wiederhold) produced CPE in cultures of human cells originating from kidney, embryonic skin-muscle, and uterine tissues. This strain also multiplied in cultures of human embryonic brain tissue. In contrast, another strain (High Point, type 4) multiplied in suspended cell cultures of human embryonic brain but not in kidney or in skin-muscle or in embryonic intestinal tissues.[4] Shaw,[5] studying strains belonging to 10 serotypes obtained growth (apparently without CPE) of types 2 (8 passages) and type 4 (including Slater and Syverton's type 4) strains in minced embryonic chick cells. Strains representing types 1, 3, 5, and 6 failed to grow. Stulberg[6] passaged type 1 virus serially in cultures prepared from skeletal muscle of infant mice; however, evidence of virus multiplication was not definitely established.

Since 1957 most of the *in vitro* studies on propagation of the Coxsackie viruses have used cells obtained from primate species. Habel and Loomis[7]

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* Professor of Research Pediatrics University of Kansas Medical Center. Formerly, Instructor, Department of Preventive Medicine, Yale University School of Medicine, 1944-1946.

** Research Assistant in Virology, Section for Virus Research.

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and Johnsson and Lundmark[8] obtained CPE with several Group A type 7 viruses on cells derived from monkeys and from human beings. Soon thereafter, Sickles *et al.*[9] while adding 5 new serotypes (types 20-24) to the Group A viruses noted that types 20, 20a, 20b, 21 and 23 produced CPE during passage on primate cells. Lehmann-Grube and Syverton[10,11] obtained in primary cultures of human amnion cells propagation with CPE for types 6, 9, 10, 11, 13, 14, 15, and 18; in 1959, Syverton sent us type 16 virus which he and his associates had also propagated *in vitro*. Recently, Dunnebacke and Maltern[12] also reported on the "adaptation" of type 10 on human amnion cells. Lenahan and Wenner[13] obtained CPE on amnion cells with types 3, 8, 12, 17, and 24. Thus, within the last 10 years reports have appeared in the literature on the propagation in tissue cultures of 22 of the 24 Group A Coxsackie viruses.

The advantages of utilizing Group A Coxsackie viruses propagated in tissue cultures for work in progress in the University of Kansas became evident during studies on preparation of type-specific reference antisera. In studies with 24 prototypic viruses we have been able to propagate, with associated CPE, 17 serotypes in either human amnionic or in other primate cells cultivated *in vitro*. This report summarizes some of the biological interactions associated with replication on mammalian cells of Group A Coxsackie viruses.

MATERIALS AND METHODS

Viruses. The 24 Group A serotypes, and 2 subtypes (types 20a and 20b)* were made available to us by Dr. Hildegaard Plager,** Division of Laboratories, New York State Health Department, Albany, N. Y. The histories of the prototypic viruses[9, 14, 15] are summarized in Table 1. Virus stocks used for inoculating tissue cultures were derived from infected mice; however, 2 serotypes (20, 20a, and 21) had been passed in tissue cultures, although subsequently each was maintained in weanling mice at Albany.

Antisera. Dr. Plager also provided type-specific antisera. Some other type-specific antisera were prepared by Syverton and associates; in addition, several prepared in this laboratory were available.

Tissue cultures. The 9 tissues used in the study were obtained from human beings, monkeys, and mice. Tissues were washed, minced, and "digested" with trypsin using

* Serotypes 20a and 20b are considered at this time to be subtypes of Group A, type 20 virus. The 26 viruses included in the study therefore have been considered as belonging to 24 serotypes.

** We are indebted to Dr. Plager and to Dr. Gilbert Dalldorf, Sloan-Kettering Institute for Cancer Research, Rye, New York, for making available all serotypes except Vispo; the latter serotype (type 23) was made available to us by Dr. Italo Archetti (Instituto Superiore di Sanità, Roma) while he was a member of the Section for Virus Research.

TABLE 1. RESUMÉ OF ORIGINS AND PASSAGE HISTORIES FOR 26 GROUP A COXSACKIE VIRUSES

Virus			*History of origin*				*Pasage in laboratory*	
Type	*Accession no.*	*Strain*	*Age, sex*	*Year*	*Kind of illness*	*Geographic area*	*N.Y.S.*	*K.U.*
1	48249	Tompkins	9, ♂	1947	P	Coxsackie, N. Y.	M_1,H_3,M_{86}	M_1
2	49190	Fleetwood	9, ♂	1947	"	Wilmington, Del.	M_{50}	M_1
3	49191	Olson	–, ♂	1948	–	New York State	M_{47}	M_1
4	50246	Ala 4-CDC	–, –	?	?	Alabama?	$>M_{18}$	M_1
5	5134	Swartz	5, ♂	1950	?	New York State	M_{17}	M_1
6	5011	Gdula	5, ♀	1949	NP	Silver Creek, N. Y.	M_{27}	M_1
7	50140	Parker	7, ♂	"	NP?	Rochester, N. Y.	M_{31}	Ha_4
8	5010	Donovan	3, ♂	"	NP	Levittown, N. Y.	M_{29}	M_1
9	50546	Bozek	6, ♀	1950	–	Glen Cove, N. Y.	M_{30}	M_1
10	50548	Kowalik	7, ♂	"	P?	New York State	M_{19}	M_1
11	52148	Belgium 1	18 mo., ♂	1951	pleurodynia	Belgium	M_{18}	M_1
12	51204	Texas 12	flies	1948	none	Texas	M_{16}	M_1
13	5359	Flores	23 mo., ?	1952	?	Mexico	M_{11}	M_1
14	52113	G 14	1¼, ♂	1950	none	S. Africa	M_7	M_1
15	52108	G 9	2, ♂	"	"	"	M_{13}	M_1
16	52109	G 10	2, ♀	1951	"	"	M_{16}	M_1
17	52111	G 12	3, ♂	"	"	"	M_{16}	M_1
18	52112	G 13	2, ♀	1950	"	"	M_{16}	M_1
19	53157	Dohi	5, ♂	1952	P	Japan	M_{23}	M_1
20	55166	IH #35	pool	1955	hepatitis	New York State	$HE_7HeLa_5M_{17}$	M_2
20a	5536	Tulane 1623	3, ♂	1951	NP?	Bayou Bouif, La.	HE_3M_{15}	M_1
20b	5721	Cecil	2½, ♂	1951	none	S. Africa	M_{19}	M_1
21	55161	Kuykendall	17, ♂	1952	P	San Leandro, Cal.	HE_4M_{17}	HA
22	5630	Chulman	14, ♂	1955	GI	New York State	M_9	M_1
23	—	Vispo	2, ♂	1955	NP	Ancona, Italy	—	MK_3HA_4
24	5720	Joseph	3, ♂	1952	none	S. Africa	M_{13}	M_1

Accession numbers are those assigned these viruses by the New York State Department of Health. Under strain: IH, infectious hepatitis. Under kind of illness: P, paralytic; NP, non-paralytic; GI, gastrointestinal; ?, unknown. Kinds of passages: M, suckling mice; H, infant hamster; HE, human embryonic tissues; MK, monkey kidney; HA, human amnion. The investigators supplying many Group A serotypes to the New York State Department of Health, as well as those recognized by Dalldorf and Sickles, have been published elsewhere.[9, 14, 15]

methods previously described.[16, 17] The media[18-23] used from growth and maintenance of each cell "type" are summarized in Table 2.

Measurements.

In tissue cultures. Each stock virus was inoculated into 3 or 4 cultures, depending on availability, using 0.1 ml inoculum per culture (volume of maintenance medium

TABLE 2. CULTIVATION AND MAINTENANCE OF TISSUE CULTURES: MEDIUMS USED IN RELATION TO APPEARANCE OF CELLS

	*Medium used**				*Appearance of cells on indicated days after inoculation***				
					No. of	*Days*			
Kind of culture	*for growth*	*pH*	*for maintenance*	*pH*	*tests*	*0-1*	*2-4*	*5-6*	*>7*
Human amnion	199, + 20% Hs	7.0	199, + 0.5% Leh	7.3	27	E	G	G	F
" foreskin	", + 10% "	7.0	", + " "	7.0	5	G	F	F	F
" lung	", + 20% "	7.0	Eagles' + 5% Ch.s	7.5	4	G	F	F	F
" kidney	LG, + 5% Cs	7.4	199, + 0.5% Leh	7.2	4	G	F	F	F
" , HeLa	YEM + 15% Hs	6.8	", + 7.5% Cs	7.0	14	G	F	F	F
" , KB	" + " "	6.8	", + 7.5% Cs	7.0	10	G	F	F	P
Monkey kidney	LG + 2% Cs	7.0	$hcAE_m$	7.4	12	E	E	E	G
" testes	B + 3% "	7.4	B + 3% Cs	7.6	13	E	E	G	F
Mouse embryo	199, + 20% Cs	7.5	199, + 2% Cs	7.6	9	G	G	F	F

* The kinds of mediums are designated by abbreviations; details concerning composition of these mediums are listed in the following references: Medium 199[18]; LG, lactalbumin growth medium[19]; YEM, yeast extract medium + tryptose phosphate[20, 21]; medium B[22]; Eagle's[23]; $hcAE_m$, high cystine altered Eagle's maintenance without any added serum.[17] Hs, human serum; Cs, calf serum; Ch.s, chicken serum; Leh, lactalbumin enzymatic hydrolysate; percentages refer to volume of serum or Leh present in medium.

** E, excellent; G, good; F, fair; P, poor. Excellent, indicates outgrowth of contiguous monolayers of cells; poor, indicates peeling of cell sheet involving more than half the surface of the culture; the cells remaining attached showed cytoplasmic granularity. Good and fair are intermediate gradings. Gradings were obtained only for uninoculated control cultures entered in tests. pH entries are initial values when medium was added to cells. Cultures were incubated at 37 ± 0.5° C.

per culture was 1.5 ml). Inoculated tubes were returned to a drum and "rolled." If CPE was not observed by the 7th ± 1 day, the fluid overlay from each culture was harvested and pooled; this material constituted the inoculum for another set of cultures of the corresponding cell type. Four or more passages were made before studies with any cell line were terminated.

After the appearance of CPE, which was sometimes incomplete at first, passage was continued until maximal CPE was obtained. At this level of passage, fluid overlays were harvested and pooled for subsequent titration and specificity studies. For titration purposes serial decimal dilutions of virus were made; each dilution was tested in 3 or 4 replicate cultures. TCD_{50} endpoints were determined by the Reed-Muench method.[24]

Serotypic specificity was ascertained by determining serum dilution endpoints (SDE's) with serotypic antisera using approximately 100 TCD_{50} of virus. Inactivated sera (56° for 30 minutes) were diluted serially in 4-fold steps; after addition of virus, mixtures were incubated (37 C.°) for 1 hour. Cultures inoculated with serum-virus mixtures were observed for 7 days, or until appropriate endpoints were obtained in control tubes inoculated with several dilutions of virus.

In suckling mice. Mouse torso suspensions which were obtained from mice previously infected with designated serotypes as well as the fluid overlays harvested from tissue cultures inoculated with corresponding serotypes were tested in newborn mice. Each undiluted or diluted stock was tested in 8 mice (litters were randomized); each mouse received 0.08 ml inoculum by intramuscular (0.04 ml) and intraperitoneal (0.04 ml) routes. Mice were observed twice daily. Scores were based on illness, paralysis and death occurring 36 hours after inoculation. One litter of uninoculated mice and one other inoculated with phosphate buffered saline were included as controls in each experiment.

Fluorescent antibody studies. Stock viruses were inoculated on monolayers of human amnion cells previously grown on glass slips in Leighton tubes. On each of 3 consecutive days infected slip cultures were removed from the tubes; each was washed twice with PBS prior to fixation in acetone. After drying (37° for 30 minutes) the slips were stored at 4° until stained. Potent antiserum (SDE $>$ 1:1000/0.1 ml) prepared in monkeys was conjugated with fluorescein isothiocyanate.[25-27] After addition of conjugated antiserum to the cell layer the slips were set (for 30 minutes) in a petri dish containing wet cotton. The antiserum was removed by washing with saline phosphate buffer, pH 7.0. The mount was viewed by ultra-violet microscopy using Corning #5840 and Wratten 2B filters, and an Osram 200 watt light source.

RESULTS

VIRUSES PRODUCING CYTOPATHOGENIC EFFECT (CPE)

In human amnion cells.

Of the 26 Group A viruses entered in the study, including types 20a and 20b, a total of 18 produced CPE on passage in primary cultures of human amnion cells. Of these, 14 viruses produced unequivocal CPE at first passage, and 4 viruses (types 3, 8, 12, and 16) either failed to cause CPE initially, or else cytopathic changes were equivocal. After several consecutive passages, however, the latter viruses caused maximal CPE. Also among these were 5 viruses which initially caused maximal cell destruction, but thereafter, displayed a lesser CPE for several passages (see *e.g.* types 11 and 17); however, these 5 viruses after several additional passages produced maximal CPE. Eight of the 26 Group A viruses have failed thus far in our studies to produce CPE in amnion cells; some of these viruses have been through 8 or more consecutive passages. Data pertaining to the 18 viruses propagable in human amnion cells are recorded in Table 3.

TABLE 3. PROPAGATION OF GROUP A COXSACKIE VIRUSES IN PRIMARY CULTURES OF HUMAN AMNION CELLS

Type	Total no. serial passages	Extent of CPE* at indicated levels of passage						Day(s) when CPE was		Titer of stock TCD_{50}/0.1 ml	Type-specific neutralization	
		1	2	3	4	5	≧6#	First observed	Maximal		SDE 0.1 ml	TCD_{50} in test
A-3	12	+[2]	+[2]	+[1]	+[2]	++[2]	++++[2]	3	5	4.7	256	250
A-8	7	0[3]	+[1]	++++[3]	++++[3]	++++[3]	++++	3	6	3.8	1024	81
A-9	4	++++[3]	++++[3]	++++[3]	++++[3]			3	3	6.5	2000	60
A-10	3	+++[3]	++++[3]	++++[3]				2	5	4.7	1024	92
A-11	4	+++[3]	+++[3]	+[3]	+++[3]			6	10	4.0	360	40
A-12	6	+[1]	++++[3]	+++[3]	+++[3]	++++[3]	++++[3]	3	5	4.7	500	368
A-13	4	+++[3]	+++[3]	++[3]	++++[3]			3	7	≧3.7	40 360	100 100
A-14	4	++[3]	++++[3]	++++[3]	++++[3]			3	3	4.3	500	2000
A-15	4	+++[3]	++[3]	++[3]	+++[3]			6	10	≧4.7	176	175
A-16	4	+[3]	+[1]	++[3]	++++[3]			3	3	3.7	500	60
A-17	8	+++[2]	0[3]	0[3]	++++[3]	++++[3]	++++[3]	3	5	3.5	630	20
A-18	4	++++[3]	++[3]	++[3]	++[3]			4	7	>5.0	2000	18
A-20	8	++[3]	+[2]	+[2]	++++[3]	++++[3]	++++[3]	5	6	5.6	400	100
A-20a	7	++++[3]	+++[3]	+++[3]	++++[3]	++++[3]	++++[3]	4	10	5.0	1610	100
A-20b	6	++++[3]	+[2]	+[2]	++++[3]	++++[3]	++++[3]	4	10	5.2	389	370
A-21	4	++++[3]	++++[3]	++++[3]	++++[3]			3	3	4.0	25	300
A-23	4	++++[3]	++++[3]	++++[3]	++++[3]			3	3	5.5	6400	320
A-24	9	++++[2]	+[1]	+[1]	0[3]	++[2]	++++[3]	3	6	4.5	64	268

* CPE, cytopathic effect; TCD_{50}, 50% infective dose for tissue culture; SDE, serum dilution endpoint. Extent of CPE is listed: +, definite focal signs of cellular destruction involving from 50 per cent ++, 75 per cent +++ to complete ++++ destruction of cell sheet. Superscript numerals 1, less than 50 per cent, 2, 50 to 75 per cent and 3, all cultures showed CPE. # refers to type of change observed at passage level entered in Column 2. Data for days of onset of CPE, etc. and titers are for stocks obtained at levels of passage entered for type in Column 2.

Other human cell cultures.

Among the 8 viruses that failed to injure visibly amnion cells, only type 7 produced CPE on HeLa cells. None of the other 7 viruses (type 1, 2, 4, 5, 6, 19, and 22) produced CPE in any of the 5 cell lines listed in Table 4.

Among the 18 viruses that caused CPE on amnion cells four serotypes (types 3, 8, 10, and 17) failed to cause CPE on any of the other human cell lines entered in the study. Others, such as types 9, 12, and 23 produced CPE on primary cultures of human cells, but not on continuous cultures of HeLa or KB cells. Two strains (types 16 and 24) were cytopathic for HeLa and KB, but were not cytopathic for kidney or skin cultures derived from human beings. The 9 remaining viruses produced CPE on all lines of human cells entered in these tests; the CPE caused by the type 14 on cultures of human skin were of an equivocal nature.

Other primate cell cultures.

From monkeys. Six prototypic viruses produced CPE on either renal or testicular cell cultures derived from monkeys. Four serotypes (types 9, 14, 16 and 23) produced CPE with both kinds of culture. Two viruses (types 7 and 10) however, produced CPE only on renal cells.

From mice. None of the 26 Group A viruses caused demonstrable CPE on cultures prepared from mouse embryonic tissues (Tables 4 and 7).

NATURE OF VIRUS PROPAGATION

Kinds of CPE.

Human Amnion cells. Two major kinds of CPE were observed. The majority of the Group A serotypes caused similar CPE. The first signs of injury consisted of "plaques" containing rounded, or dendritic cells with granular cytoplasm. The rates with which CPE advanced varied for different serotypes (see Table 3). At the final stage most cells were sequestered, rounded and dark in appearance. These rounded cells sloughed from the glass, whereas some of the elongated type remained adherent. Several serotypes (*e.g.* types 9, 20a, 21, and 23) produced CPE like that of polioviruses. CPE advanced rapidly after onset; within 24 to 48 hours the sequestrated cells lysed and fell from the glass, leaving nothing more than granular debris. The differences between these 2 kinds of CPE on amnion cells are illustrated in Figure 1.

Human skin. At onset of CPE, cells sequestrated, became rounded and shrunken. The cytoplasm appeared granular. Sequestrated cells either lysed or dropped off the glass surface leaving behind a granular residue.

TABLE 4. RANGE OF CPE OBTAINED FOR 19 GROUP A COXSACKIE VIRUSES PASSAGED IN 7 KINDS OF TISSUE CULTURE. EXTENT OF CPE AT DESIGNATED LEVELS OF PASSAGE AND YIELD OF VIRUS AT LEVEL OF MAXIMAL CPE

Group A Coxsackie serotype	*Human*																				*Monkey*										*Mouse*			
	Kidney CPE				*Skin CPE*				*HeLa CPE*					*KB CPE*							*Kidney CPE*					*Testis CPE*					*Embryo TC CPE*		*Newborn*	
	Passage		*Day*		*Passage*		*Day*		*Passage*		*Day*			*Passage*		*Day*					*Passage*		*Day*			*Passage*		*Day*			*Passage*			
	1	*4*	*O*	*M*	*1*	*4*	*O*	*M*	*1*	*4*	*O*	*M*	TCD_{50}	*1*	*4*	*O*	*M*	TCD_{50}			*1*	≧*4*	*O*	*M*	TCD_{50}	*1*	*4*	*O*	*M*	TCD_{50}	*1*	*4*	LD_{50}	
3	0	0	–	–	0	0	–	–	0	0	–	–	–	0	0	–	–	–			0	0	–	–	–	0	0	–	–	–	0	0	8.7	
7	0	0	–	–	0				+	+++	2	2	3.5	0	0	–	–	–			+	++++	4	4	4.7	0	–	–	–	–	0	0	7.3	
8	0	0	–	–	0	0	–	–	0	0	–	–	–	0	0	–	–	–			0	0	–	–	–	0	0	–	–	–	0	0	8.3	
9	++++	++++	2	2	0	±	–	–	0	0	–	–	–	0	0	–	–	–			++++	++++	2	2	6.5	++++	++++	2	3	4.7	0	0	7.0	
10	0	0	–	–	0	0	–	–	0	0	–	–	–	0	0	–	–	–			0	++++	2	7	2.8	0	0	–	–	–	0	0	8.0	
11	++++	++++	2	5	++	++++	2	3	+	++++	4	4	4.5	+	++++	3	3	1.5			0	0	–	–	–	0	0	–	–	–	0	0	7.6	
12	++++	++++	2	5	0	0	–	–	0	0	–	–	–	0	0	–	–	–			0	0	–	–	–	0	0	–	–	–	0	0	7.6	
13	++++	++++	2	5	++++	++++	2	2	++++	++++	4	4	2.7	++++	++++	3	3	3.5			0	0	–	–	–	0	0	–	–	–	0	0	7.9	
14	++++	+	5	7	0	±	–	–	++++	++++	4	4	4.0	+	++++	3	3	5.0			0	++++	2	5	4.5	+	++++	2	2	3.5	0	0	7.5	
15	++++	++++	2	5	++++	++++	3	6*	++++	++++	4	4	2.7	++++	++++	3	3	3.3			0	0	–	–	–	0	0	–	–	–	0	0	7.5	
16	0	0	–	–	0	0	–	–	+	++++	4	4	3.7	+	++++	3	8	3.3			+	++++	2	5	3.5	+	++++	3	6	3.7	0	0	–	
17	0	0	–	–	0	±	–	–	0	0	–	–	–	0	0	–	–	–			0	0	–	–	–	0	0	–	–	–	0	0	6.7	
18	++++	++++	2	5	++++	++++	2	3	++++	++++	4	4	5.3	++++	++++	3	3	3.0			0	0	–	–	–	0	0	–	–	–	0	0	7.5	
20	++++	++++	2	5	++++	++++	2	3	++++	++++	5	5	3.7	++++	++++	4	4	3.7			0	0	–	–	–	0	0	–	–	–	0	0	5.9	
20a	++++	++++	2	5	++++	++++	2	3	++++	++++	5	5	4.9	+++	+++	4	6	1.5			0	0	–	–	–	0	0	–	–	–	0	0	6.6	
20b	++++	++++	2	5	+	++++	2	2	++	++++	5	5	4.3	++	+++	4	6	2.3			0	0	–	–	–	0	0	–	–	–	0	0	4.8	
21	++++	–			++++	++++	3	3	+++	++++	2	4	4.5	++++	++++	3	3	3.3			0	0	–	–	–	0	0	–	–	–	0	0	–	
23	++++	–			+	++++	3	3	0	0	–	–	–	0	0	–	–	–			++++	++++	2	2	5.7	++++	++++	3	3	4.7	0	0	5.8	
24	0	0	–	–	0	0	–	–	+++	++++	2	7	<1.0	+	+	4	6	2.5			0	0	–	–	–	0	0	–	–	–	0	0	6.1	

Infectivity endpoints are expressed as reciprocals of $\log_{10}$ values per 0.1 ml inoculum; O, day CPE first observed; *M*, day when CPE was maximal. For grading of extent of CPE see Table 3, ±, equivocal CPE, not fully substantiated; 0, CPE not observed; —, not done. Since types 1, 2, 4, 5, 6, 19, and 22 failed to produce CPE in these cell lines, they are omitted from the table. Although most viruses were passaged only 4 times, some have had additional passages, hence ≧4.

* $TCD_{50} = 10^{-6.5}$/0.1 ml.

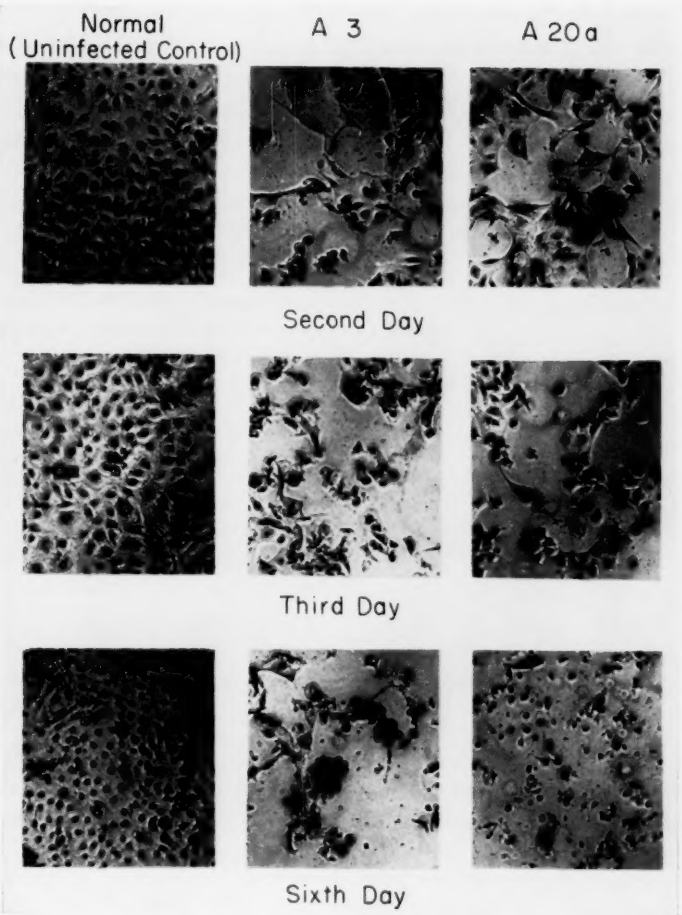

FIG. 1. Kind of cytopathic effect produced on primary cultures of human amnion cells by Group A, types 3 and 20a Coxsackie viruses. From cells grown on glass slips. Unstained. 100x. Days refer to period of time after cultures were inoculated with A3 and A20A viruses. The appearances of uninoculated cultures prepared from the same amnion, and photographed at intervals corresponding with infected cultures, are illustrated in the figure.

Human kidney. The reaction was similar to that obtained in human skin.

HeLa and KB cells. The kind of CPE resembled that generally obtained in human amnion cultures.

Monkey kidney and testis. Onset of CPE was associated with appearance of "plaques"; CPE advanced at irregular rates (Table 4); eventually the rounded cells lysed or fell away from the cell sheet. For types 10, 14, and 16 rounded cells remained attached to the glass longer than others; for types 7, 9, and 23, CPE was rapid with extensive lysis of cells.

TABLE 5. COMPARATIVE SENSITIVITIES OF FOUR CULTURAL SYSTEMS FOR MEASURING CONCENTRATIONS OF INFECTIOUS VIRUS

Group A Coxsackie serotype	*Infected fluid overlay from*	*TCD_{50} in cultures of*		*Infected fluid overlay from*	*TCD_{50} in cultures of*		
		type of origin	*amnion*		*amnion*	*MK*	*KB*
10	MK	2.8	4.3	amnion	4.7	3.2	<1.0
11	KB	1.5	3.3*	"	4.0	<1.0	nd
16	—	nd	nd	"	3.7	2.5	2.5*
18	KB	3.0	2.3*	"	≧5.0	nd	1.7*
24	HeLa	<1.0	<1.0	"	4.5	<1.0	2.5*

* Incomplete CPE in cultures inoculated with 10 to 100 TCD_{50}.

Infectivity titers.

The titers (TCD_{50}/0.1 ml) obtained in fluid overlays harvested from cultures showing CPE are entered in Tables 3 and 4. The titers obtained from amnion cells ranged between $10^{8.5}$ (type 17) to $10^{9.5}$ (type 9). Lower titers (often >10 fold) were often obtained with fluids harvested from infected cultures of KB, HeLa, and monkey testicular cells inoculated with the same serotype. On the other hand, only one (type 10) of the 6 serotypes producing CPE on renal cells (Table 4) gave a titer significantly lower than was obtained in amnion cells.

Specificity of viruses

Each Group A virus propagated in amnion HeLa, KB, and monkey renal or testicular cultures, was neutralized by homologous type-specific antisera. The SDE's obtained for Group A viruses propagated in amnion cells appear in Table 3.

Comparative sensitivities of cell lines for quantitative studies

The data summarized in Tables 3 and 4 indicated that infectivity measurements varied independently for several different serotypes (*e.g.* types 15 and 24) and for the cultural system in which they were propagated. Group A viruses producing CPE in *primary cultures* originating from human and simian sources provided, with but several exceptions, similar infectivity endpoints. Most TCD_{50} endpoints ranged in values within experimental error in these relatively crude assays. However, yields obtained with types 11

TABLE 6. COMPARATIVE LD_{50} ENDPOINTS OBTAINED IN MICE FOR SIX VIRUSES BEFORE AND AFTER PASSAGE IN AMNION CELLS

Group A Coxsackie serotype	*Mouse torso*		*Infected amnionic fluid*				
			4th passage		*last passage*		
	Mouse LD_{50}*	*Amnion TCD_{50}*	*Mouse LD_{50}*	*Amnion TCD_{50}*	*No. passages*	*Mouse LD_{50}*	*Amnion TCD_{50}*
3	8.7	<1.0	1.1	nd	12	≤0.7	4.7
8	8.3	<1.0	<1.0	"	7	<0.3	3.8
12	7.9	<1.0	2.6	"	6	≥2.0	4.7
14	8.2	2.8	1.3	4.3	4	1.4	4.3
17	6.8	1.4	<1.0	nd	8	≥1.0	3.5
24	6.9	1.4	<1.0	"	9	<0.3	4.5

All endpoints were determined on basis of 0.1 ml inoculum.
* Mouse LD_{50} values may differ from entries in Table 4 because several different stock virus pools were used for passage in tissue cultures.

and 20a in KB, and with type 24 in HeLa cells were significantly lower than for other cell lines entered in the study.

In order to explain whether the differences were due to inefficiency of virus replication, or insensitivity of the cultural systems 5 serotypes producing CPE in 4 cultural systems were retested in heterologous tissue culture systems. The results of these tests appear in Table 5. Some cell types (HeLa and type 24) were incapable of producing the same amount of virus produced at the same level of passage in amnion cells. Other cell types (renal cells and type 10) were capable of such production. But, in general, and with an appreciation of the inequities of systems employed in these tests, amnion cells almost always provided a better substrate than others both for growth and for measuring maximally the amounts of virus present in fluid overlays.

PATHOGENICITY FOR MICE OF GROUP A VIRUSES PROPAGATED IN AMNIOTIC CELLS

Group A viruses propagated in human amnion cells acquired significant reduction in virulence for mice. By the 4th passage high concentrations of infected tissue culture fluids were required in order to cause either paralysis or death in 50 per cent or more of mice. Additional passages of these viruses in amnion cells failed to significantly change the LD_{50} endpoints. At the last passage levels given in the table the number of viral particles required

TABLE 7. FAILURE TO DETECT CPE IN NINE KINDS OF TISSUE CULTURES DURING SERIAL PASSAGES OF SEVEN GROUP A COXSACKIE VIRUSES
(Origin, infected mouse torso.)

	Kind of culture								
	Human						*Monkey*		*Mouse*
*Type**	*amnion*	*kidney*	*skin*	*lung*	*HeLa*	*KB*	*kidney*	*testis*	*embryo*
A1	0	0	0	0	0	0	0	0	0
A2	0	0	0	0	0	0	0	0	0
A4	0	0	0	0	0	0	0	0	0
A5	0	0	0	0	0	0	0	0	0
A6	0	0	0	0	0	0	0	0	0
A19	0	0	0**	0	0	0	0	0	0
A22	0	0	0**	0	0	0	0	0	0

* These serotypes have been passaged serially in human amnion, 8x; mouse embryo, 5x; human lung, 3x; and for all others, 4x. Another attempt to obtain CPE for these viruses was made by adding virus to cells in suspension. These exposed cells formed monolayers without plaque formation or occurrence of CPE.
** Questionable CPE at 4th passage on monolayers of human skin.

to produce disease in mice ranged between 320 and 10,000 TCD_{50} (Table 6). The reduction in virulence for mice of type 12 was less than for the 5 other viruses entered in the tests.

Titers of virus derived from infant mice and titrated in these animals were almost always greater than titers derived from amnion cells and titrated also therein. Amnion cells were not as sensitive as suckling mice for measuring the virus "load" present in infected mouse torso (Table 6).

GROUP A VIRUSES NOT YET ESTABLISHED IN TISSUE CULTURES

The 7 Group A viruses which have not produced CPE in tissue cultures thus far are types 1, 2, 4, 5, 6, 19, and 22. These serotypes have now been passaged at least 3 and as many as 8 times in the 9 cultural systems listed

in Table 7. Excepting for very questionable onset of CPE in cultures of human skin with types 19 and 22 the results have been uniformly negative.

In order to determine whether virus replication occurred without causing CPE in cultures of human amnionic and monkey renal cells, fluid overlays obtained with these 7 and with 16 other Group A viruses were repassed in several heterologous cellular systems. These same fluids were also tested in suckling mice. The fluid overlays obtained in passage of these 7 viruses on human amnion or in monkey renal cell cultures likewise failed to cause CPE on KB, HeLa, renal, and testicular cell cultures derived from monkeys, and mouse embryo tissue cultures. The results of these tests are recorded in Table 8.

OTHER TESTS IN TISSUE CULTURES AND SUCKLING MICE

Fluids harvested from infected monkey kidney and mouse embryo cells were also passed on human amnion cells; similarly, fluids harvested from HeLa, KB, monkey testicular, and mouse embryo cells were repassed on monkey renal cells. In this way evidence of viral multiplication in monkey renal cells was obtained for types 10, 14, and 16 when the fluid overlays were passed in human amnion fluid. Subsequently these viruses consistently produced CPE in monkey renal cells.

The type 1 virus after 5 passages on mouse embryonic tissue cultures, and the type 6 virus after 8 passages on amnion cells, even in the absence of CPE, produced illness and death in suckling mice. The type 1 virus produced paralysis and death in 35 suckling mice; extracts from carcasses of these mice produced a similar fatal illness. Although inoculation of suckling mice with fluid overlays from 4th, 7th, and 8th passages of type 6 produced illness and death (31/63 mice), segmental limb paralysis of questionable nature was either transient in duration or was not observed. Extracts prepared from carcasses of mice which had developed illness after inoculation with fluid overlays obtained from amnion cells (type 6 virus) on further subinoculation produced a paralytic type of illness causing death of mice.

The use of immunofluorescence for demonstration of virus multiplication in cultures of cells which failed to show CPE did not reveal the presence of labelled virus antigens. None of the 7 serotypes following "blind" passages on mouse embryonic or on human amnion cells was specifically labelled. Preliminary studies using the indirect method of immunofluorescence has not exposed intracellular virus particles for type 1 grown on mouse embryonic, or for type 6 grown on human amnion cells.

TABLE 8. PASSAGE OF 23 GROUP A COXSACKIE VIRUSES FOLLOWING FOUR OR MORE TRANSFERS ON PRIMARY AMNION OR MONKEY KIDNEY CELLS IN SEVERAL OTHER KINDS OF TISSUE CULTURE AND IN SUCKLING MICE

		Passaged primarily in						
		amnion			*MK*			
		and repassaged in						
Group A Coxsackie serotype‡	*CPE* on amnionic cells*	*MK*	*Mo Embr*	*SM*	*Mo Embr*	*M testis*	*KB*	*HeLa*
1	0	0	0	0	0	0**	0	0
2	0	0	0	±	0	±	0	0
3	+	0	0	+	0	0**	0	0
4	0	0	0	0	±	0**	0	0
5	0	0	0	0	0	nd†	0	0
6	0	0	0	+	0	0	0	0
8	+	0	0	±	0	0	0	0
10	+	+	0	nd	0	nd	nd	nd
11	+	0	0	"	0	"	"	"
12	+	+	0	+	0	"	"	"
13	+	0	0	nd	0	"	"	"
14	+	+	nd	+	nd	"	"	"
15	+	0	"	nd	"	"	"	"
16	+	+	"	"	"	"	"	"
17	+	0	0	+	0	0	0	0
18	+	0	0	nd	0	nd	nd	nd
19	0	0	0	0	0	0	0	0
20	+	0	0	nd	0	0	nd	nd
20a	+	0	0	"	0	0	"	"
20b	+	0**	0	"	0	0	"	"
21	+	0	0	"	0	0**	"	"
22	0	0	0	0	±	0	"	0
24	+	0	0	±	0	0**	0	0

* Abbreviations: CPE, cytopathic effect; MK, monkey kidney; Mo Embr, mouse embryo; SM, suckling mice; M, monkey; KB and HeLa established cell lines. Amnion cells were primary outgrowths obtained from human amnionic membranes. Symbols: 0, 0/4 cultures showed CPE; +, 4/4 cultures showed CPE; ±, 2 or 3 of 4 cultures showed questionable CPE.

** Probably negative although 1/4 cultures showed questionable CPE.

† nd, not done.

‡ Types 7, 9, and 23 are not entered in the table; they produce CPE on amnionic and monkey renal cells; undiluted fluid overlays produce paralysis and death in suckling mice.

DISCUSSION

Our interest in propagating the Group A Coxsackie viruses in tissue cultures was aroused when the University of Kansas was asked to prepare type-specific antisera. At the start of the work in 1958 a study was made to determine the number of prototypic strains which could be propagated on cells cultivated *in vitro*. By that time several types (*e.g.*, 7, 9, 20, 21, and

TABLE 9. THE PRESENCE OR ABSENCE OF VIRAL ANTIGENS IN TISSUE CULTURES IN WHICH CPE WAS NOT REVEALED

Group A Coxsackie serotype	*Tested in suckling mice*		*Mouse embryo TC 4th passage**	*Tested by immunofluorescence*
	amnionic			
	4th passage	*8th passage*		
1	neg	neg	+	0
2	"	±**	neg	0
4	"	neg	"	0
5	"**	"	"**	0
6	+?	+?	"	0
19	neg	neg	"	0
22	"	"	"**	0
14	+	nd	nd	+

* Mouse embryo TC, 4th and 5th passage fluid overlays (type 1 virus) produced paralysis and death in 3 independent tests (death rate 35/35). Type 6 fluid overlays between the 4th and 8th passages killed 31/63 mice in 4 independent tests.

** Scattered deaths without observance of paralysis, deaths were usually ≤30 per cent of litter; occasionally an entire litter died or was cannibalized during the first 10 days after inoculation. The type 14 virus was cytopathic on amnion cells (see Table 6); it was used as a positive control in these tests.

23) were known to cause CPE on primate cells (2, 3, 7, 9, 10). Syverton and his associates were studying the problem too; we received types 10, 11, 13, 15, 16, and 18 viruses adapted to human amnion cells[10,11] from the University of Minnesota laboratories.* Subsequently, as reported in this paper we were able to propagate not only the same serotypes as they did,

* The late J. T. Syverton sent us all prototypic Group A strains which he and his associates cultivated in amnion cells. He also sent us type-specific antisera which he and his associates had prepared. The early experiences with these reagents were of material help in pilot studies preliminary to the final steps in antiserum production. We are grateful for this assistance. Later we obtained corresponding serotypes from Dr. Plager. The tissue culture "adapted" viruses reported here represent stocks obtained in this laboratory with prototypic mouse-adapted viruses.

but to add 5 more, namely types 3, 8, 12, 17, and 24[18] to the list of Group A viruses propagable on primate cells in culture.

The addition of 5 new serotypes to the 12 previously known to be cytopathic leaves 7 Group A viruses which in our studies have not yielded CPE in tissue cultures. Undoubtedly these 7 serotypes, when conditions are fulfilled, will be propagable in tissue cultures. Several possible explanations might apply to these rejections. First, the virus population maintained continually in mice for many passages could have provided a population of viruses with very limited tropism for primate cells. We obtained several serotypes recently recovered from human beings; these strains had been passed only a few times in mice. They also failed to produce CPE. Moreover, all these viruses rejected mouse embryonic cells, just as they had primate cells. Another explanation, suggested by the implicit evidence obtained for latent infection with type 1 virus in mouse cells, and possibly for type 6 virus in amnion cells, is that some factor may be missing in the substrate which is essential for maximal growth and cytopathogenicity of these viruses. These singular viruses may form a stratum within the Group A complex requiring an essential but still unrecognized metabolite.[28] Certainly for serotypes such as type 3 some unknown requirement had to be fulfilled during passage before complete CPE was achieved.

Several of our Group A viruses following serial passage in amnion cells were less virulent for mice than the infected mouse tissues from which they were derived. When comparable virus preparations from cultures at different passage levels were titrated in mice the gain in infectivity for the culture system was associated with a decreased pathogenicity for mice. Our results were very similar to those obtained by Lehmann-Grube and Syverton.[11] These authors, on the basis of more extensive observations than ours believe that during "adaptation" mutations which occur at a high rate lead to variants with decreased mouse virulence and increased tropism for cells in cultures. Our studies do not add further insight into the phenomena responsible for these qualitative changes in viral character.

A greater effort was made to obtain CPE with amnion than with other primate cells entered in the study. Human amnion cells had been successfully used before. The other cell lines were used with differing frequencies: those less frequently used (*e.g.*, human skin and kidney) were less readily obtainable than others. When it became evident that amnion cells were more sensitive to CPE than other cell lines, further passages in the latter systems were often discontinued. These different systems were utilized however for more extensive studies among the 7 serotypes not yet yielding CPE.

The use of tissue cultures for studies with the Coxsackie viruses has advantages over the use of suckling mice. Studies on epidemiology and pathogenesis can be implemented and expanded. Antigens obtained in fluid overlay can be used in neutralization and very probably in complement fixation tests. Viruses, "adapted" to tissue cultures retain serological specificity and thereby facilitate assay measurements in serological studies. The system partially eliminates the use of suckling mice for many biological determinations. The latter system is fraught with difficulties in bio-assays. But it would be unwise to predict that suckling mice will be eliminated in all studies for as yet this living organism appears to be more sensitive than amnion or monkey renal cells cultured *in vitro* for the detection on primary passage of many Group A viruses present in feces and other body exudates.

SUMMARY

Of the serotypic Group A Coxsackie viruses, representing the prototypes catalogued by Dalldorf and Sickles,[9,14] 17 have been propagated in tissue culture. All but one of these 17 strains produced CPE in primary human amnion cells; the one strain (type 7) which thus far has failed to affect amnion cells produced CPE in renal cells obtained from monkeys. Several of these 17 viruses also produced cytopathic changes on human kidney and skin, and on HeLa, KB, and monkey renal and testicular cells. Among these 17 Group A viruses there are 5 serotypes which have been identified recently[18] as capable of causing CPE in tissue cultures. A total of 7 serotypes presently listed among the prototypic Group A Coxsackie viruses were not associated with cytopathic changes following at least 4 passages in 9 tissue culture systems. Type 1 virus and possibly type 6 virus may have propagated without producing CPE in several independent cultural systems. However, additional studies are necessary in order to eliminate seriate carry-over of residual virus.

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